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Recent Advances in the Search for Synthetic Antimalarials*,†

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Nearly a half-century has passed since the disclosure of the essential details of the life history of the malarial plasmodium and its mode of transmission by mosquitoes. At the turn of the century it was thought by many that our knowledge of the disease was then sufficiently great to insure the early removal of malaria as a world problem. It appeared at that time that widespread administration of quinine for the treatment of existing cases of the disease, plus the use of efficient larvicides and insecticides against the mosquito vectors, was all that would be required to establish control of malaria. That we have fallen far short of such optimistic predictions is only too evident. Actually, according to Coggeshall (1), malaria as a world disease probably was increasing in intensity even prior to the onset of the present war. Some idea of the peacetime magnitude of the problem in our own country may be envisioned from estimates (2) which indicate an average of 4,000,000 cases a year and 4000 deaths, accompanied by an annual economic loss not less than $500,000,000. In tropical regions, where malarial surveys often indicate the rate of infection to be 100% of the population, the resulting economic loss is almost impossible to estimate. According to Hehir (3) the eradication of malaria in India, whose people at present suffer from an estimated 100,000,000 cases of the disease annually, would in a single generation convert that country into one of the most prosperous in the world.

In time of war, as in peace, malaria has always been one of the most, if not the most, important of all medical problems. Coggeshall (4) has reviewed the part played by malaria in World War I, and has given some indication of its grave effects in the present conflict. It has been estimated that at the time we lost Bataan, 85% of our troops were suffering from this disease. Malaria is prevalent in many of the islands of the Southwest Pacific, and the infection rate among army personnel in that region has exceeded 50%. The over-all picture from the various war areas indicates that probably more cases of the disease are appearing among our troops per day than we experienced per year in the last war. At present no reasons exist for expecting this condition to improve.

Warnings also have been issued relative to the possible spread of malaria in the postwar period (4, 5, 6). Because we lack drugs capable of effecting a radical cure of malaria, relapses of the disease are frequent and conditions of latency may persist for long periods of time. This fact, combined with the high infection rate prevalent

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among many of our troops, makes it appear inevitable that large numbers of men will be returned to this country bearing active or chronic malarial infections. The most important mosquito vector for this country, *Anopheles quadrimaculatus*, is distributed widely as far north as the Canadian border. With a population almost nonimmune to malaria, certainly nonimmune to the foreign strains of the parasite which would be introduced, and with an abundance of a proper mosquito vector for the transmission of the parasites, it is not unlikely that the return home of infected troops may be followed by epidemics of the disease.

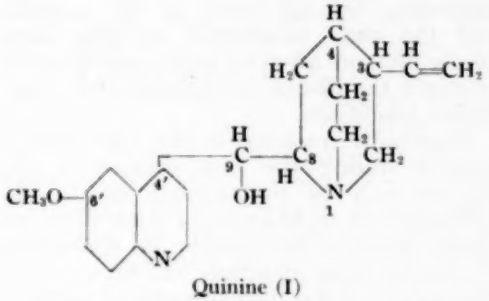
Even in the absence of a state of war, Coggeshall (4) has pointed out that the changing circumstances of the times, especially as reflected in the speed and volume of modern transportation, makes it possible for travelers to acquire malarial infections and still return home before the incubation period of the disease has elapsed. In this way cases of malaria may be transported back to regions previously free of disease. In addition, the speed of modern transportation, especially by air, may result in the introduction of mosquito vectors especially efficient as transmitters of the disease. The most noteworthy example of the result of such an event is the epidemic of malaria that occurred in Brazil in 1938 following the accidental introduction of *Anopheles gambiae*, the most notorious of the African vectors of malaria. Soper and Wilson (7) quote official estimates of over 100,000 cases and a minimum of 14,000 deaths during the first six months of that year, and relate the dramatic story of the subsequent eradication of this species of mosquito from Brazil by the joint efforts of the Brazilian Government and the International Health Division of the Rockefeller Foundation. It is quite evident that, barring the development of truly superior antimalarial drugs prior to the termination of hostilities, research in this field of chemotherapy must be continued in the postwar period.

Attempts to control malaria by drug treatment have failed because of a lack of therapeutic agents capable of acting as true causal prophylactics, or ones capable of producing a radical cure of the infection. However, even in the presence of such deficiencies, Boyd (8) and Russell (9) have questioned whether the present status of malaria is justifiable in view of the wealth of information that has been accumulated on methods of controlling the mosquito vector. Efficient methods exist for the control of the larval or adult stages of the insect through drainage and manipulation of water, oiling, dusting with Paris green, screening of dwelling places, and use of pyrethrum sprays, and the brilliant results of antimosquito campaigns in the Canal Zone, the Malay states and Brazil have demonstrated their practical value. Boyd (8) and Russell (9) conclude that failure to extend such campaigns is due in the main to certain social obstacles. These include, among others, such social factors as an absence of public knowledge as to the economic importance of malaria, ignorance of or apathy to the demonstrated value of mosquito control on the part of public officials, and fears as to the real or imagined cost of these campaigns. Since these and similar factors have hindered the adaptation of mosquito control measures in even the more enlightened sections of the world, the difficulties encountered in trying to apply them in tropical regions are easily understood. Although in any case at least partial control of the insect vector probably will be required, final solution of the malaria problem may have to await the development of drugs capable of acting as true preventatives of the disease, or at least of preventing relapse following cure of the acute infection.

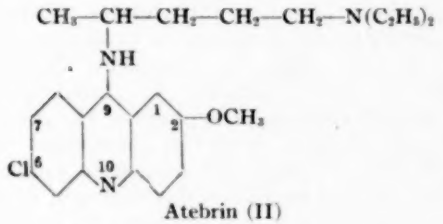
During that part of its life cycle spent within the human host, the malaria parasite potentially is susceptible to attack by chemotherapeutic agents at a number of stages. At the time of biting, an infected mosquito injects the form of the parasite known as a sporozoite. Experience to date has demonstrated that the antimalarials now in common use, which for all practical purposes may be limited to quinine (I), atebrin (II) and plasmochin (III) or drugs closely related to these compounds, are unable to act on this form of the parasite. A possible exception to this generalization is plasmochin, for which some action against sporozoites is claimed, although only in doses approaching the toxic limit for humans.

The fate of the malaria parasite, between the time of its injection as a sporozoite and its appearance some ten days or more later as an occupant of the red blood corpuscles, is still a subject of controversy. The most recent view (10, 11), as based mainly upon research in avian malaria, pictures the sporozoites as entering cells of the reticuloendothelial system, and there initiating a cycle of asexual reproduction. According to this theory, these nonpigmented endothelial or exo-erythrocytic parasites likewise are resistant to the action of quinine, atebrin or plasmochin, again with the possible exception of the last-named drug. Toward the end of the incubation period of the disease, from this endothelial cycle forms are

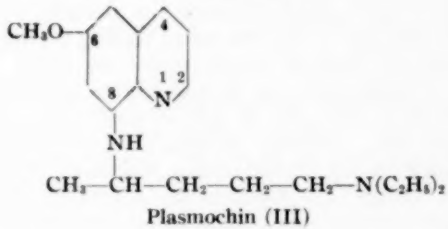
given off which enter erythrocytes and develop into the pigmented parasites commonly seen on examination of blood smears from infected vertebrate hosts. It is against the two types of these erythrocytic parasites that our present-day antimalarial drugs are effective. One form of the parasite, known variously as a trophozoite, schizont or merozoite, depending upon its stage of development, multiplies within the red blood corpuscles by an asexual process, and is responsible for the clinical symptoms of the acute infection. Quinine and atebrin are effective against these parasites, and



Quinine (I)



Atebrin (II)



Plasmochin (III)

drugs resembling them in action are termed schizonticides. The second type of erythrocytic parasite may be differentiated into male and female forms, known collectively as gametocytes. These are not concerned with the disease process in the human, being present in relatively few numbers in comparison with the schizonts, but are the forms by which malaria is transmitted from the vertebrate host to an uninfected mosquito at the time of biting. The male and female gametocytes combine and undergo a process of sexual reproduction within the mosquito, which results in the release of sporozoites in the body cavity of the insect. Some of these sporozoites make their way to the salivary glands, and the mosquito is then ready to infect a new vertebrate host. Gametocytes present in the blood of the human host are susceptible to plasmochin, and drugs similar to this compound are said to be gametocidal in action.

It is thus apparent that the blood stream infection in the vertebrate host can be controlled by the use of schizonticidal drugs such as quinine and atebrin, and gametocidal drugs such as plasmochin and compounds related to it. The distinction as to type of action is not entirely definite. Schizonticidal drugs act also to some extent upon gametocytes, while compounds primarily gametocidal also effect schizonts in some degree. From the review on the action of drugs in avian, simian and human malaria by Curd (12), it is apparent that the degree of crossing of gametocidal and schizonticidal activity in any drug may be dependent both on the species of parasite and the particular vertebrate host concerned.

Theoretically it would appear that if every human case of malaria in the world received during a given period adequate medication with a schizonticidal drug and with a gametocidal drug, such as a combination of quinine with plasmochin, it should be possible to disrupt the man-mosquito cycle of the parasite, and the incidence of the disease should drop at once. That such a procedure would fail to eliminate malaria is due to the tendency of the disease to relapse. Quinine and atebrin control the blood stream infection, but they do not always sterilize it. The occurrence of relapses over a variable period of time permits the accumulation of large reservoirs of cases infective for mosquitoes.

The mechanism of relapse in malaria is not well understood. Again the most modern view (10, 11) is based upon the existence of the parasites in cells of the reticulo-endothelial system, as determined experimentally in avian malaria. According to this theory the endothelial parasites, which are resistant to the action of known drugs, can outlast for a greater or lesser time the parasites residing in the erythrocytes, and can always give rise again to an infection of the blood stream. Thus, although parasites in the blood may be destroyed by the action of quinine, atebrin and plasmochin the endothelial parasites survive and can again cause an infection of the erythrocytes. Eventually, according to this theory, the immunity mechanisms of the vertebrate host develop to a degree where they can eliminate the infection in the endothelial cells, and final cure results.

Chronologically, attempts to solve the problem of malaria control can be classified in an approximate manner into three periods. Following the demonstration of mosquito transmission of the disease, it was natural that during the first quarter of the present century interest should be centered around attempts to control the insect vector. Techniques for use in antimosquito campaigns developed rapidly, and many notable achievements, such as that of Gorgas in the Canal Zone were recorded. However, as has been mentioned already, certain social obstacles have hindered adequate utilization of these processes in spite of their demonstrated usefulness (8, 9).

Early in the century Rabe (13) published the formula for the cinchona alkaloids (I), and this disclosure provided the impetus for research on the synthesis of many derivatives of these compounds, as well as of quinolyl ketones and alcohols resembling them in structure. The results of this work, and similar later efforts, have been reviewed by Henry (14,15) and by von Oettigen (16). Though a great deal of research of this kind was done, it did not result in the discovery of any product significantly superior to quinine in its activity.

Accurate determination of the antimalarial efficacy of such compounds was difficult due to an inability to cultivate the malaria parasite *in vitro*, and because of a lack of a suitable *in vivo* biological test. In many instances the compounds were compared with quinine as to their antipyretic action, or comparative toxicities to certain protozoa were determined. Such methods are of little value for the selection of possible antimalarial drugs. Bass and Johns (17) in 1912 attempted the *in vitro* culture of the malarial plasmodium, but their results, and those of later workers of that period, demonstrated that the organisms could be maintained at best for only a few generations. Many attempts were made to transmit human malarial parasites to all classes of vertebrates, but in no case was it found possible to establish a stable infection. The literature on the use of rabbits, guinea pigs, hamsters, dogs, rats, mice, bats, chimpanzees and monkeys in these experiments has been reviewed by Taliaferro and Cannon (18). The most favorable result was the temporary establishment of *Plasmodium falciparum* in monkeys.

During this time research was being carried on by the Sergent brothers, Kopanaris, Marks, Brunn and others on the possible use of bird malaria as an agent for the determination of the antimalarial activities of drugs. Their work, culminating in the announcement by Roehl (19) in 1924 of a standardized method for the estimation of antimalarial activity based on canary malaria, has been summarized by Fourneau, *et al.* (20) and by Marshall (21). The introduction of Roehl's method of testing placed research on synthetic antimalarials on a scientific basis.

Another discovery which was to influence the course of malaria research was that of Wagner-Jauregg (22), who demonstrated the beneficial effects of the disease in the treatment of human cases of syphilis of the central nervous system. Wagner-Jauregg's method consisted in infecting paralytics with blood containing asexual forms of the parasite, and the wide adaptation of this therapeutic measure afforded many opportunities to study the disease in humans under controlled conditions.

Essentially, progress in the fight against malaria during the first quarter of the century may be summarized as: (*a*) development of techniques for controlling the insect vector of the disease; (*b*) elucidation of the chemical structure of the cinchona alkaloids and the development of methods of synthesis of compounds related to them; (*c*) evolution of a means of evaluating the activities of drugs by comparing their effects on avian malaria; (*d*) introduction of malaria therapy of syphilis of the central nervous system, with its attendant opportunities to study the disease in humans.

The second period of development of research on malaria and methods for its control covers approximately the decade from 1925 to 1935. By the use of testing methods based on various avian malarias, much of the earlier work on the cinchona alkaloids and their derivatives was re-evaluated and extended. Among others, reports were published by Boyd (23), Giemsa, Weise and Tropp (24), Giemsa and Oesterlin (25), Fourneau and his collaborators (26), Goodson, Henry and Macfie (27), and Buttle, Henry and Trevan (28). Mention has been made of reviews on this work (14, 15, 16), and of the fact that it failed to result in the discovery of any derivative markedly superior to quinine in activity. For the greater part, the variations in the cinchona structure (I) which were studied were alterations in the chemical nature of the vinyl group at position 3 of the quinuclidine ring, of the secondary alcohol group at position 9, and of the alkoxy group at position 6′ of the quinoline ring, along with substitution of various groups at the 5′ and 8′ positions of this ring. In addition attempts were made to correlate variations in the four centers of asymmetry, positions 3, 4, 8 and 9, with

antimalarial activity. In general, the levorotatory alkaloids were found to be morel active in bird malaria than their dextrorotatory isomers. Thron and Dirscherl (29) observed that epimerization at carbon atom 9 yielded derivatives which were inactive in bird malaria. Aside from these observations, the most striking result of this work was the demonstration of the importance of the secondary alcohol grouping, any chemical change at this position resulting in compounds with no antimalarial activity.

The most important result following upon the development of Roehl's method for testing drugs was the introduction of plasmochin (III) by Schulemann and his coworkers (30), and, subsequently, the announcement of atebrin (II) by Mauss and Mietzsch (31). Since the discovery of these drugs an exceedingly large number of derivatives similar to them in constitution have been synthesized. In the plasmochin series, research groups led by Fourneau in France, by Magidson and Kritchevskii in Russia and by Robinson and Kermack in England, have been especially active. Reviews covering this field have been published by von Oettigen (16), Henry (14), Fischl and Schlossberger (32), Findlay (33), Bishop (34) and Curd (12). In the synthesis of compounds related to atebrin, special mention is to be made of the work done by the Russians under the leadership of Magidson. Reviews on such derivatives have been made available by Curd (12), Henry (14), Findlay (33) and Bishop (34).

The results of these investigations have resembled those obtained in attempts to modify the structure of the cinchona alkaloids; they are of considerable scientific interest, but they have not yielded products possessing antimalarial activities significantly greater than those of atebrin and plasmochin. Because of this fact, and since reviews on such research are available, only a brief summary of the results obtained will be given here.

In the plasmochin series (III), investigations in the main have dealt with the effects of (*a*) modification of the alkoxy group in position 6, (*b*) variation of the alkylamino side chain at position 8 with regard to length, degree of branching and the introduction of other substituents, (*c*) modification of the terminal dialkylamino group of the side chain and (*d*) change in the position of attachment of the side chain.

The experimental testing of these compounds has involved the use of certain variations of Roehl's original method. In that method, the effect of drugs on canaries infected with the malarial plasmodium is determined. In such infections, both schizonts and gametocytes are present in the blood. If a drug has a beneficial effect on the disease, it is assumed to be schizonticidal in action, since only the schizonts or asexual forms of the parasite are concerned with the development of the acute infection. In order that the gametocidal activity of drugs might be determined, the use of Haemoproteus infections in the Java sparrow was introduced. In the case of these parasites, which are quite closely related to those causing malaria in birds, only the gametocytes appear in the peripheral blood, the schizonts undergoing their cycle of development in endothelial cells. A drug active in removing parasites from the blood in Haemoproteus infections in the Java sparrow is, therefore, gametocidal in effect. Discussions of this and other variations in the technique of testing antimalarial compounds have been given by Curd (12), Henry (14), Marshall (21), Bishop (34) and Hewitt (35). In general, no drug is purely schizonticidal or gametocidal in its action. The degree to which it possesses each type of activity may depend upon both the species of parasite and the particular vertebrate host employed. In human malaria, plasmochin essentially is gametocidal in action, but in avian malaria it possesses in addition schizonticidal activity to a varying degree.

As determined in various avian malarias, as well as Haemoproteus infections in the Java sparrow, the effect of an increase in the molecular weight of the alkoxy group in position 6 of the plasmochin type (III) of compound is to lower both gametocidal and schizonticidal activity. Activity is retained to some extent when the methoxyl group is replaced by hydrogen or hydroxyl, but not when substitution is by a methyl group.

Increasing the length of an unbranched diamine side chain at position 8 of the quinoline ring has an effect on both gametocidal and on schizonticidal activity. With a side chain of the type $—NH—(CH_2)_n—N(C_2H_5)_2$, maximum gametocidal activity is obtained when $n = 5$, while a maximum in schizonticidal activity is observed when $n=9$. In the case of either gametocidal or schizonticidal action, compounds having side chains containing an odd number of carbon atoms show the higher level of activity. In general, branching of the carbon chain was found to be dystherapeutic in effect, as was true also of the attachment of hydroxyl groups or introduction of ether links into the chain proper. Variation in the nature of the terminal dialkylamino group is permissible, although maximum activity generally is found with $—N(C_2H_5)_2$.

In the same way that increase in the length of the diamine side chain at position 8 causes a shift from gametocidal toward schizonticidal activity, change of the point of attachment of the side chain from position 8 to position 4 of the quinoline ring is followed by a similar alteration in therapeutic effect (36).

In the atebrin series (II) of compounds, increase in the length of a side chain of the type $-NH-(CH_2)_n-N(C_2H_n)_2$ at position 9 does not result in an alteration of activity dependent upon whether n represents an odd or even number. Schizonticidal activity rises up to a maximim at $n = 4$, and then drops with further increase in chain length. In general, branching of the chain is dystherapeutic in effect.

Increase in the weight of the alkoxy group at position 2 lowers activity, as does replacement by a methyl radical. Introduction of an additional methoxy group at position 3 destroys activity. Substitution of a methyl mercapto group at position 2 lowers the effectiveness of the compound.

Shifting the halogen from position 6 to 7 lowers activity, as is true also when chlorine atoms are substituted at both positions 6 and 7. Replacement of the chlorine atom at 6 by a nitro group yields an inactive compound, though substitution of such a group at position 7 yields a derivative with moderate antimalarial activity.

The literature contains hundreds of references relating to the clinical usage of atebrin and plasmochin. Many of these have been summarized in the Fourth General Report of the Malaria Commission of the League of Nations (37). In brief it has been found that atebrin is at least as effective as quinine as a schizonticide, though, like quinine, it has only a weak action on gametocytes. It is not effective as a causal prophylactic. Plasmochin, by virtue of its weak schizonticidal activity, has not been found useful in the treatment of the acute infection. It is especially effective as a gametocide, and thus it complements quinine and atebrin in activity. Given in association with either of these drugs, plasmochin generally is considered to have a favorable effect on the rate of relapse. James (38) found plasmochin to possess some value as a causal prophylactic. However, the toxicity of the drug has prevented its use for such a purpose.

Introduction of the malarial therapy of paresis, as induced by either the inoculation of infected blood or by the bite of an infected mosquito, offered many opportunities to study the immunology of the disease. Along with corresponding investigations of avian and simian malaria, many references to such work may be found in the literature. Reviews covering this field have been made by Redmond (39), Taliaferro (40) and Coggeshall (41). In brief, it has been found that immunity in malaria is: (*a*) relatively weak in action and transitory in duration; (*b*) highly specific both as to the species and strain of parasite involved; and (*c*) directed toward the blood stream infection rather than toward sporozoites. Also it has been demonstrated that both cellular and humoral immunity mechanisms are involved, and that one is relatively ineffective in the absence of full development of the other.

To the progress of research in the chemotherapy of the disease, the most important result of studies on the malarial therapy of paresis was the demonstration of the differences that exist between malaria induced by the inoculation of infected blood (artificially induced malaria), and that which follows biting by infected mosquitos (naturally induced malaria). As has been summarized by Boyd (42), Kikuth (43) and others, artificially induced malaria is characterized by: (*a*) complete suppression of the incubation period of the disease when massive doses of schizonts are used in the inoculation; (*b*) ready response clinically or prophylactically to treatment with schizonticidal drugs such as quinine or atebrin; and (*c*) relative freedom from relapse following termination of the acute infection by therapy with these drugs. On the other hand, malaria resulting from inoculation by sporozoites, either through the medium of the mosquito or by injection of a suspension of the parasites, is characterized by: (*a*) a more or less constant minimum incubation period regardless of the dose of sporozoites employed; (*b*) ready response clinically but not prophylactically to quinine or atebrin, although plasmochin is able to exert some prophylactic action; and (*c*) a marked tendency to relapse following cure of the acute infection by quinine or atebrin, although again plasmochin appears to have a somewhat favorable effect on such an event.

With general observations of this type available to him, James (38) in 1931 suggested that sporozoites on entry into the vertebrate host underwent a cycle of development in tissue cells, such as those of the reticulo-endothelium, before they reached the circulation and entered erythrocytes. The necessary intermediation of a cycle of this sort would explain the inability to shorten below a certain minimum the incubation period in naturally induced malaria. If these tissue cell parasites also were resistant to the action of drugs, and

could outlast those residing in the erythrocytes, their existence could offer an explanation for the tendency of naturally induced malaria to relapse. Several years thereafter Huff and Bloom (44) and Raffaele (45) announced the discovery of a tissue cell or exo-erythrocytic cycle of development of certain avian malarial parasites.

The developments of this second period in the search for better methods of malaria control can then be summarized as: (*a*) evaluation of the antimalarial activities of the cinchona alkaloids and their derivatives; (*b*) discovery of atebrin and plasmochin and the synthesis of a number of derivatives similar to them; (*c*) determination of the clinical and prophylactic value of the atebrin and plasmochin types of drugs; (*d*) elucidation of the main characteristics of malarial immunity; and (*e*) development of a theory to account for the differences between artificially induced and naturally induced malaria, along with tentative substantiation of the theory by virtue of the demonstration of residence of certain avian malarial parasites in tissue cells.

The third period of research on the problem of malaria extends from 1935 up to the present time in 1944. From the material which has been published in the literature, it would appear that the most important advances which have been made are concerned with an extension of our knowledge of the mechanism of infection in the vertebrate host, along with increases in information as to the biology of the malarial parasites.

Soon after the demonstration of exo-erythrocytic schizogony by Huff and Bloom (44) and Raffaele (45), adequate confirmatory reports appeared in the literature. In excess of a hundred, such publications have been reviewed by Porter and Huff (46). German workers in particular, led by Kikuth (10), were active in developing theories as to the significance of these endothelial parasites for the mechanism of infection in the animal host. A summation of these theories already has been presented. Following injection by the bite of an infected mosquito, sporozoites are pictured as entering cells of the reticulo-endothelium, and there initiating an asexual cycle of reproduction. Forms are given off which enter red corpuscles and develop into ordinary pigmented parasites. Therapy of this blood stream infection by quinine or atebrin results in the removal of the erythrocytic parasites, but those residing in the tissue cells survive by virtue of their resistance to these drugs. When suitably stimulated, they can again cause an infection of the blood stream, and the host suffers a relapse. Missiroli (47) advocates still another stage of development for the sporozoites prior to their entry into tissue cells or erythrocytes.

Infection mechanisms such as the one outlined above still are a subject of controversy, but there exists a growing tendency to assume that this scheme of development, or one similar to it, represents the course of the disease in the human as well as the avian host. Direct evidence of the penetration of sporozoites into tissue cells and subsequent development into exo-erythrocytic schizonts has not yet been obtained. However, the occurrence of such forms in certain of the avian malarias is established beyond dispute, and Porter and Huff (46) have reviewed the evidence for the presence of similar endothelial parasites in monkey and human malaria. Kikuth (43) has shown that in the case of *P. cathemerium* sporozoite-induced infections in canaries, after forty-eight hours the presence of unpigmented parasites in cells of the reticulo-endothelium could be demonstrated, while pigmented forms in the red blood corpuscles could not be found until much later. Further he has demonstrated quinine and atebrin to be without effect on the exo-erythrocytic parasites, while plasmochin was found to possess such action in a moderate degree. This ability of plasmochin to act on the endothelial parasites agrees with the favorable effect of the drug on the relapse rate in human malaria, and with its partial effectiveness as a causal prophylactic. Adler and Tchernomoretz (48) demonstrated that the administration of quinine, in a daily dose of 150 mg./Kg. of body weight, eliminated the pigmented erythrocytic forms of *P. gallinaceum* from the blood stream of fowls, but left the exo-erythrocytic schizonts intact. Cessation of quinine treatment was soon followed by the appearance of erythrocytic parasites.

In certain types of avian malaria, exo-erythrocytic parasites develop regardless of whether infection is by blood inoculation or by injection of sporozoites (49). Since in bird malaria chronic infections may result from either type of inoculation, such a result is not inconsistent with the theory relating endothelial parasites to relapse. However, since chronic infections rarely result in the case of artificially induced human malaria, it is assumed that exoerythrocytic parasites arise in the human host only after the injection of sporozoites.

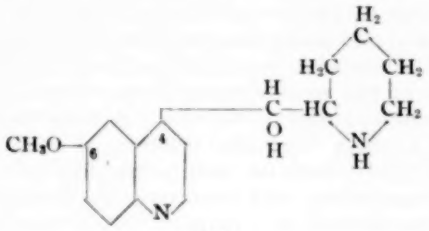
During this period research on the plasmochin and atebrin types of compounds has continued, but no new derivatives of par-

ticular clinical effectiveness have been developed. Attention also has been directed toward attempts to determine the mode of action of atebrin and plasmochin on the malaria parasite. Marshall (21) has reviewed the literature on this subject. Because of past difficulties in demonstrating an *in vitro* action of the drugs on the parasites, except in concentrations much higher than could be maintained in the blood stream, an indirect attack of these compounds on the malarial plasmodium formerly was postulated. More recently Beckman (50) and Boyd and Dunn (51), among others, have shown that the therapy of avian malaria by quinine, atebrin or plasmochin results in a partial inhibition of reproduction by the parasites, and causes a reduction in the size of the schizonts so that fewer or greater cells are produced. Morphological changes in the parasites are detectable. Bock and Oesterlin (52), with the aid of fluorescent microscopy, demonstrated that atebrin accumulated rapidly within the parasites, and that such accumulation always could be detected before any morphological changes appeared. Hewitt and Richardson (53), in 1943, stated that whatever the mode of action of quinine, atebrin and plasmochin might be, the end result is degeneration of the malaria parasite. Such an effect was demonstrated by the changed appearance of the parasites during treatment, and by the delayed infection curves that were observed when blood from treated birds was used as the inoculum for uninfected hosts. From all available evidence it would appear that quinine, atebrin and plasmochin exhibit a direct plasmodicidal effect.

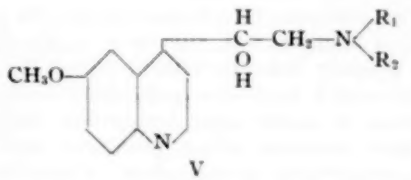
Since considerable interest always is attached to the synthesis of simpler structures which retain the pharmacological activity of the parent naturally occurring alkaloids, the work of Ainley and King (54) and King and Work (55, 56) should be mentioned. Ainley and King (54) found 4-(6-methoxyquinolyl)-α-piperidyl carbinol (IV) to have a toxicity of the order of quinine, along with approximately half the antimalarial activity of that drug. Removal of the methoxy group destroyed activity, as did alkylation of the piperidyl nitrogen atom.

In a further simplification, King and Work (55) found 6-methoxy-4-quinolyl carbinols of the structure (V) to be antimalarial in action when R_1 and R_2 both represented butyl, amyl or hexyl groups. Antimalarial activity was lost when groups having molecular weights outside this range were employed. Further, King and Work (56) demonstrated that when R_1 represented a hexyl group, and R_2 either an ethyl, propyl, butyl or amyl grouping, inactive compounds were obtained. Work (57) also attempted to replace the methoxyquinoline portion of such compounds by aromatic nuclei, or by aliphatic chains of sufficient length to bring the molecular weight into the range 300–400, but no derivative with antimalarial activity resulted.

Both Rabe and his co-workers (58, 59) and Prelog and his associates (60), synthesized cinchona alkaloids (I) in which the vinyl group at position 3 was lacking. Prelog



IV



V

(60) tested a racemate isolated from the reaction mixture, and found it to possess antimalarial activity. From this he concluded that the vinyl group in position 3 is not essential for action on malaria parasites. Rabe (58) isolated the four theoretically possible isomers, but failed to observe any activity among them on avian malaria. Later (59) it was reported that activity did exist for one of the two racemates of these isomers.

In addition, some progress has been made in the discovery of structures, other than those resembling quinine, atebrin or plasmochin, which possess antimalarial activity. Glyn-Hughes, Lourie and Yorke (61) found 1:11-*n*-undecane diamidine to have some action on human malaria. Christophers and Fulton (62) observed a similar effect in simian, but not in avian malaria. Other diamidines have been found partially effective in human and in avian malaria (63, 64).

Sulfanilamide, and some of its derivatives, has been found to be active against avian (65, 66), simian (67) and human malaria (67). While such compounds have not been found to be especially effective in

avian or human malaria (67), the ability of sulfanilamide to produce a radical cure of *P. knowlesi* infections in monkeys has attracted attention. Ability to cure without subsequent relapse is rare, and it is to be hoped that a sulfanilamide derivative with comparable activity in human malaria will be found.

The desirability of having available a method for the *in vitro* cultivation of malarial parasites has resulted in the stimulation of further research in this field. Niyogi and Roy (68) have studied the cultivation of *P. knowlesi* by a modified Bass and Johns (17) method. The cultures remained infective for monkeys for four days. Trager (69) has worked with the avian malaria parasite, *P. lophurae*. To his basic culture medium various growth factors were added. Calcium pantothenate was found to influence markedly the survival of the parasites. Under the most favorable conditions, *P. lophurae* was kept viable for about two weeks. As a result of studies made with *P. lophurae* and *P. cathemerium* infections in normal and biotin-deficient ducks and chickens, Trager (70) has offered a qualified opinion that biotin is an essential growth factor for malarial parasites with a certain optimal range of concentration. He has pointed out that further research is required to establish this point.

Manwell and Hewitt (71) cultivated at least one generation of *P. praecox* in capillary tubes, while Hewitt (72) had the same degree of success with *P. cathemerium* planted on an inspissated egg and rabbit serum medium. Wolfson (73) successfully cultivated several avian malarial parasites in duck embryos, and reports (74) cultivation of the exo-erythrocytic stages in tissue culture.

Christophers and Fulton (75) initiated studies on the respiration of malarial parasites by means of the Warburg respirometer. This work has been extended by Velick (76), Fulton (77), Wendel and Kimball (78) and Maier and Coggeshall (79, 80). Oxygen consumption of the parasites at various growth stages, and utilization of certain sugars by the organisms, has been determined. Antimalarial drugs added to the parasite suspensions decreased oxygen consumption, but such an effect could not be correlated uniformly with the *in vivo* therapeutic activities of these compounds.

Because of the quinine shortage resulting from the loss of the East Indies to the Japanese, and more especially because of the inability of quinine, atebrin or plasmochin to act as safe and efficient prophylactics against the disease, the search for superior antimalarial drugs has been intensified since the onset of the war. Under the leadership of the National Research Council and various governmental agencies, a systematic program of research is in operation involving the co-operation of many chemical and biological laboratories throughout the country. Though the results being obtained under this program for obvious reasons cannot be published at this time, some information concerning them has been made available by Coggeshall (1). Thus it has been revealed that testing laboratories, each capable of handling as high as 200 new compounds per month, are in operation. Furthermore, facilities are available for assaying not only the therapeutic value (action on blood stream parasites) of the compounds submitted, but also the ability of these compounds to act on sporozoites and hence to serve as causal prophylactics. The magnitude of the program can be visualized from the report that during the period from 1941 to 1943, more compounds were tested than the combined total number of all tried in previous efforts. Of even greater significance is the statement that antimalarial activity is being discovered in many totally different chemical groups.

Obviously, to be complete, any attempt to review recent advances in synthetic antimalarials should include the data being accumulated under this program, since even such discreet statements as those of Coggeshall (1) make clear their importance. In the absence of this information, an attempt has been made to summarize the developments of the present century in regard to knowledge of the malarial plasmodium and methods for its control.

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Water-in-Oil Emulsifying Agents

II. Synthesis of Some Cholesteryl and Cetyl Esters*

By E. L. Cataline, Lee Worrell, S. F. Jeffries† and S. A. Aronson‡

In the first paper of this series (1) the relative efficiencies of certain components of wool fat and some analogous compounds as water-in-oil emulsifying agents with petrolatum were determined. The compounds of particular interest were the cholesteryl esters of lauric, palmitic and stearic acids which, employed in 3% concentration with the same concentration of cholesterol, were found to be capable of emulsifying 600%, 700% and 800% of water, respectively. In view of these results it seemed desirable to prepare a number of related esters and to investigate their emulsifying efficiencies.

Procedures which have been commonly used for the preparation of compounds of the type under discussion include: (*a*) the direct method which involves heating the appropriate acid and alcohol to a relatively high temperature in an atmosphere of carbon dioxide or other inert gas (1, 2, 6); (*b*) interaction of an acid chloride with an alcohol or alcoholate, usually at an elevated temperature; and (*c*) reaction of the metal salt of an acid with an alkyl halide (3, 6). Recently, Bell and Taub (4, 5) have prepared a number of esters of 12-hydroxystearic acid by refluxing the acid with the appropriate alcohol, benzenesulfonic acid or *p*-toluenesulfonic acid being used as catalyst. Because the preparation of some of the esters which we hoped to study involved the employment, as reactants, of compounds which are damaged by excessive heat, and because of the difficulty of obtaining the derivatives demanded by some of the procedures mentioned above, the last-named method was selected for further study.

In the study of this procedure two considerations of major importance presented themselves, namely, the relative proportions of the reactants to be employed and, since most of the reactants employed were solids, the solvent in which the reaction was to be carried out. In a preliminary series of experiments in which the relative proportions of the reactants were varied and different anhydrous solvents employed, the data shown in Table I were obtained. The preparation of cholesteryl stearate was selected for study because the ester is well characterized and excess reactants are rather easily removed from the reaction mixture. It is apparent that of the three solvents studied benzene was most satisfactory, toluene somewhat less so and ether quite unsatisfactory. As was presumed, use of an excess of the alcohol resulted in a higher yield than that obtained when equimolecular quantities were employed in the preparation of most of the esters mentioned below. When an excess of the acid was employed the yield approximated that obtained using equimolecular quantities. The increased solubility of the ester in the presence of the free acid and resultant loss during purification may explain this apparent anomaly. As catalyst, *p*-toluenesulfonic acid was preferred to benzenesulfonic acid because of the greater hygroscopicity of the latter.

TABLE I.—EFFECT OF VARIATION OF SOLVENT AND RELATIVE PROPORTIONS OF REACTANTS ON THE YIELD OF CHOLESTERYL STEARATE

Solvent	Molecular Ratio Cholesterol:Stearic Acid	Yield (%)
Ether	1.5:1	4
	1:1	3
	1:1.5	2
Benzene	1.5:1	48
	1:1	43
	1:1.5	42
Toluene	1.5:1	36
	1:1	32
	1:1.5	32

Page and Rudy (6) stated that they were able to prepare both the mono- and dicholesteryl esters of oxalic and succinic acids by heating the acid chlorides with cholesterol, and those of adipic and succinic acids by heating the free acids with cholesterol. Attempts to prepare, by the method described herein, mono-cholesteryl oxalate, mono-cholesteryl adipate, mono-cetyl oxalate, mono-cetyl succinate and mono-cetyl adipate were unsuccessful. It is of interest to note that the mono-cholesteryl adipate described by Page and Rudy melted at 144–145° C. while the melting point of cholesterol is variously stated as 146.5° C., 146–147° C., 147.5° C. and 148° C. (7).

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When cholesterol and adipic acid were allowed to react, in the presence of *p*-toluenesulfonic acid, we obtained the di-ester and a considerable quantity of cholesterol was recovered. These considerations seem to cast some doubt upon the identity of Page and Rudy's compound. Mono-cholesteryl succinate was obtained in small yield when equimolecular quantities of cholesterol and succinic anhydride were employed in the procedure described in this paper.

It was hoped that cholesteryl and cetyl esters of some hydroxylated fatty acids might be prepared for emulsifying efficiency studies. However, attempts to prepare such compounds by a variety of methods have yielded, with one exception, more or less gummy products which could not be characterized. Only cetyl-12-hydroxy stearate (4) has been obtained by the present method. The corresponding cholesteryl ester has so far resisted all attempts at purification.

TABLE II.—ESTERS OF CHOLESTEROL AND CETYL ALCOHOL[a]

No.	Acid	Yield (%)	M. P. (° C.)	References
	Esters of Cholesterol			
1.	*n*-Butyric	66	102 (110)[b]	(6, 8)
2.	*n*-Caproic	75	98–99	(6, 8)
3.	Lauric	46	91–92	(6)
4.	Myristic	46	80 (86)[b]	(6)
5.	Palmitic	40	89–90[c]	(6)
6.	Stearic	43	82–83	(6)
7.	Oxalic (di-ester)	88	226–227[d]	(6)
8.	Succinic (mono-ester)	28	175–175.5	(6)
9.	Succinic (di-ester)	29	220 (240)[b,e]	(6)
10.	Adipic (di-ester)	28	195 (222)[b]	(6)
	Esters of Cetyl Alcohol			
11.	Lauric	30	40–41	[f]
12.	Myristic	35	47–48	(9)
13.	Palmitic	61	53–54	(2, 10, 11, 13)
14.	Stearic	61	56–57	(12, 13)
15.	12-Hydroxystearic	70	68–69	(4)
16.	Oxalic (di-ester)	56	56.5–57.5	(14)
17.	Succinic (di-ester)	53	58.5–59	(3, 15)
18.	Adipic (di-ester)	75	56.5–57	(14, 16)

[a] Compounds 1, 2 and 3 were recrystallized from acetone, 4 and 5 from acetone or ether, 6, 11, 12, 13, 14, 15, 16, 17 and 18 from ether, 8 and 9 from dioxan, 7 from anhydrous ethyl acetate and 10 from methylethyl ketone.

[b] Like many other cholesteryl esters, these melt to a cloudy liquid at the first temperature and the liquid clears at the second temperature, often with a display of colors (*cf.* Ref. 8).

[c] With preliminary softening.

[d] Page and Rudy (Ref. 6) reported 220–230° C. with decomposition.

[e] Page and Rudy (*Ibid.*) reported 220° C. (235° C.).

[f] Saponification number: Calcd., 132.3. Found, 132.9, 133.2.

EXPERIMENTAL

Preparation of the Esters; General Procedure.—A mixture composed of 0.02 mole of the acid, 0.02 mole of the alcohol (for the preparation of di-esters of the dicarboxylic acids, 0.04 mole of alcohol was used), 0.0015 mole of *p*-toluenesulfonic acid and 150 cc. of anhydrous benzene was placed in the apparatus described by Bell and Taub (4) and refluxed for three hours in an oil bath the temperature of which was maintained between 130–140° C. The reaction mixture was allowed to cool, filtered, and the solvent removed by heating under reduced pressure on the steam bath. The residue was washed with two or three 20-cc. portions of acetone, the latter removed by suction and the crude ester recrystallized from a suitable solvent until the melting point was constant. Two recrystallizations were usually sufficient.

Mono-cholesteryl Succinate.—For the preparation of this ester succinic anhydride was used and the residue remaining after removal of the benzene was washed thoroughly with water, then with acetone and recrystallized.

Di-cholesteryl Succinate.—This ester was formed when 0.04 mole of succinic anhydride reacted with 0.02 mole of cholesterol. After the benzene had been removed the residue was washed once with acetone and once with ethyl acetoacetate and then recrystallized.

Di-cholesteryl Adipate.—The residue remaining after the removal of the benzene was washed with acetone and then extracted with 150 cc. of hot alcohol. Upon cooling there separated 3.8 Gm. of white crystalline material, m. p. 145–146° C. When mixed with an authentic sample of cholesterol there was no depression in the melting point. This represents 49% of the amount of cholesterol used.

The alcohol-insoluble portion was recrystallized from methylethyl ketone. Yield and melting point are listed in Table II.

SUMMARY

The preparation of a number of esters of cholesterol and cetyl alcohol has been described and some of their physical characteristics tabulated.

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Isopropyl Alcohol as an Extractive Menstruum for Digitalis*

By Harold F. Chase, Arnold J. Lehman and E. R. Crandall with the assistance of Henry Schwerma and Robert O. Bauer†

Isopropyl alcohol has been used for a number of years with the sanction of N. N. R. for external applications and is particularly well known as an ingredient of rubbing alcohol compound. It has been shown to have solvent properties similar to ethyl alcohol (1) and to be approximately twice as toxic (2). It has, moreover, been certified for human enteral administration in countries other than our own (3). With the increased demands for ethyl alcohol in the war effort the question arises as to whether isopropyl alcohol might be substituted as an extractive medium for medicinal products whose active ingredients are alkaloids, glucosides or other active substances and whose doses are measured in minims. This problem has already been answered in part by Lauwaet (4), who found that extracts of equal or greater alkaloidal content are obtained by using 60% to 70% isopropyl alcohol in place of ethyl alcohol of equal strength, when extracting belladonna, aconite, nux vomica and cinchona. For further investigation of this problem two extracts of powdered digitalis leaf, one with ethyl alcohol and the other with isopropyl alcohol as menstruum, were prepared. These were analyzed gravimetrically for extractive residue and bioassayed by the official U. S. P. XII cat method, the pigeon emetic and the pigeon minimum fatal dose methods.

EXPERIMENTAL

Preparation of Extracts.—The liquid extracts of digitalis were prepared by the U. S. P. XII process for Tincture of digitalis from a specimen of powdered dried leaves of *Digitalis purpurea* (Penick & Company, 1939). We quote from the label:

> "The Whole Leaf Digitalis from which the powder was milled assayed 100%. After sifting, freed from stems and powdered, the potency assayed 68%. Sampled and assayed by Dr. James C. Munch of Temple University."

The contents of the bottle were mixed and a sample taken. The extracts were prepared in such a manner that the extractive material from 100 Gm. of drug was contained in 700 cc. of solution, presuming the potency of the crude drug to be 68% of the U. S. P. standard as stated on the label. Thus, each cubic centimeter represented the soluble substances from 142.85 mg. of crude drug. Extract I was made with the official solvent of ethyl alcohol–water 4:1 by volume, representing about 75% by volume of ethyl alcohol. Extract II was prepared with isopropyl alcohol–water 4.1 by volume as menstruum and contained 75% to 80% by volume of isopropyl alcohol.

Extractive Matter.—Granting that the solvent properties of the two alcohols are similar, there still may be minor differences in the amount and character of the extractives dissolved by each, which might explain any differences in their therapeutic activity. The weight of extractive contained in each of the preparations was determined, therefore, by drying at 100° C. in accordance with the U. S. P. method for "Diluted Alcohol-soluble Extractive." The percentages contained in the ethyl and isopropyl extracts were 35.7% and 30.3%, respectively, based upon the 142.85 mg./cc. of the powdered drug, as described in the foregoing paragraph under "Preparation of Extracts." Thus the isopropyl alcohol menstruum takes up approximately 15% less extractive than does the ethyl alcohol menstruum. As an additional check these samples were incinerated, as specified in the U. S. P., for determination of the total ash. The amount of ash from the isopropyl product was 1.45% of the weight of the crude drug originally extracted, or about half of the weight of ash from the ethyl alcohol preparation (2.86% of the original sample), suggesting a difference in the solution of inorganic salts.

Bioassay.—The potency of the standard and test solutions was investigated by the pigeon minimum emetic dose, the pigeon minimum fatal dose, and the official U. S. P. XII cat methods of bioassay. The pigeon methods (5) were utilized because of the close relationship of the M Em D to the human therapeutic dose and to obtain the emetic–toxic (fatal dose) ratios. Hanzlik's original technique was followed with one minor variation: M Em D and M. L. D. doses were calculated at 50% instead of at 60%. The pigeon emetic assay involved the administration of the extracts in doses ranging from 0.1 to 0.25 cc./kg. and from 0.1 to 0.20 cc./kg. for the ethyl and isopropyl extracts, respectively (Fig. 1); the M Em D's were 0.165 cc./kg. and 0.118 cc./kg., respectively. Thus, by this method, the isopropyl alcohol product proved to be 28.5% stronger than its ethyl alcohol counterpart.

In order to obviate any question regarding the emetic action of isopropyl alcohol as such in pigeons, five times and ten times the alcohol content of the 100% emetic dose were administered to six birds, three on each dose. These were single doses of 70% isopropyl alcohol diluted one-half with saline, as is the practice in the pigeon assay. They were given intravenously within one minute. None of these birds vomited or showed any of the usual symptoms of salivation or nausea. Evidently isopropyl alcohol adds very little, if at all, to the emetic action of the digitalis. Hanzlik (5) arrived at a similar conclusion regarding ethyl alcohol.

The range of doses for the determination of the pigeon fatal dose lay between 0.5 and 1.0 cc./kg. for the ethyl and 0.5 and 0.9 cc./kg. for the isopropyl solution (Fig. 1). The M. L. D.'s were 0.806 cc./kg. and 0.712 cc./kg. In this instance the test extract was stronger by 11.7%.

As a final check on the pharmacologic activity the U. S. P. XII intravenous cat method was util-

* This report is part of the project which involves a complete pharmacologic investigation of isopropyl alcohol and is supported by the Standard Alcohol Company of New York, through courtesy of Mr. James Park.

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ized. The official technique was adhered to as closely as possible. The scarcity of animals, however, protracted the test beyond the prescribed period of time and led to the inclusion of two cats of weight greater than the limits advised. Each preparation was assayed on ten cats on the basis of an estimated fatal dose, after preliminary assays, of 0.55 cc./kg. The average number of doses of the extract with the official menstruum was 14.1, and for that with the menstruum under examination was 14.5 from the same end point, the death of the animal, they would seem to be comparable. The third relationship, a 28.5% greater potency as measured by the pigeon emetic method, may possibly be explained by the supposition that the lower proportion of extractive which was present in the isopropyl solution allows the active constituent to assert its therapeutic (emetic) action at a lower dosage level without interference from extraneous, though possibly chemically related, substances. At the toxic (fatal dose)

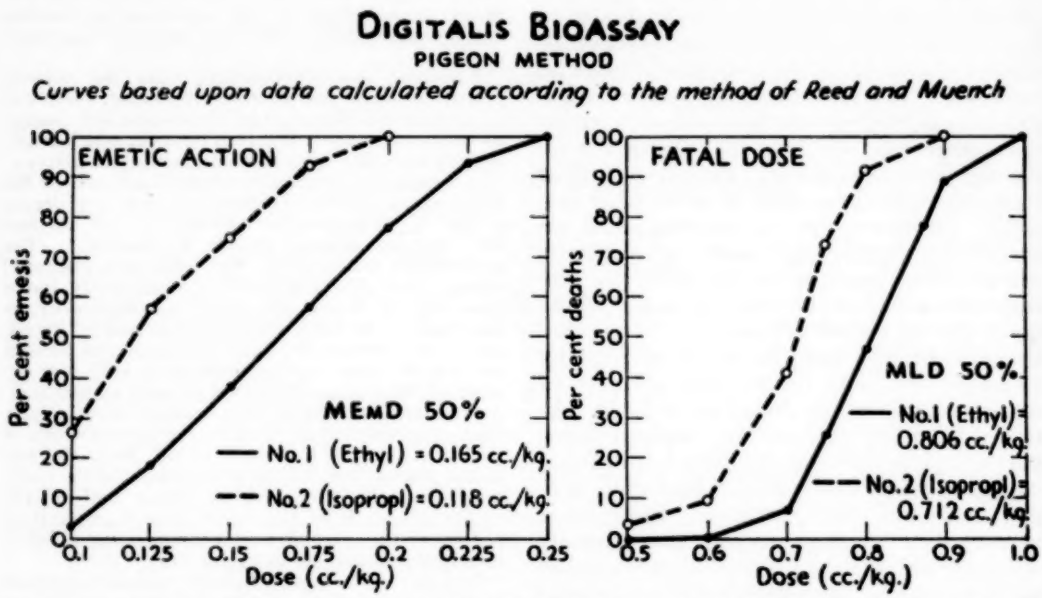

Fig. 1. Bioassay of Ethyl and Isopropyl Alcoholic Liquid Extracts of Digitalis by the Pigeon Emetic and Fatal Dose Methods.
The data are calculated according to the method of Reed and Muench (6).

TABLE I.—DIGITALIS BIOASSAY—CAT METHOD

	Prep. I (Ethyl)			Prep. II (Isopropyl)			
Cat	Wt., Kg.	No. of Doses	Fatal Dose, Cc./Kg.	Cat	Wt., Kg.	No. of Doses	Fatal Dose, Cc./Kg.
I	2.59	17	0.624	H	2.45	14	0.514
M	2.15	16	0.587	K	2.13	16	0.587
N	2.04	14	0.514	L	4.22	17	0.624
V	2.84	13	0.477	P	2.45	14	0.514
W	2.33	13	0.477	R	2.60	14	0.514
X	4.14	15	0.550	S	2.80	15	0.550
AB	2.44	14	0.514	T	2.25	15	0.550
AC	2.91	15	0.550	U	3.72	14	0.514
AD	2.09	13	0.477	Y	2.37	15	0.550
AF	2.45	11	0.407	AH	2.64	18	0.660
Average		14.1	0.518 ± 0.054			14.5	0.558 ± 0.042

(Table I). Similarly the average fatal doses were 0.518 ± 0.054 cc./kg. and 0.558 ± 0.042 cc./kg., respectively. The isopropyl alcohol solution, by this method of assay, proved to be 7.7% less active than the ethyl alcohol solution.

Comment.—We have obtained values by three methods of assay for the relative potency of two liquid extracts of digitalis. Two of these, the official cat method and the pigeon fatal dose method, show the isopropyl alcohol extract to be 7.7% weaker and 11.7% stronger, respectively, than the standard of comparison. These values are within the limits of the experimental error allowable in bioassays of this nature. Since they are derived level, the absence of these extraneous extractives might not be so great a factor. Hence, we may conclude that the isopropyl alcohol extract of digitalis is equivalent to or slightly greater in potency than that prepared by the official menstruum.

The emetic–toxic (fatal dose) ratio for the pigeon assays demonstrates a factor of safety of 4.5 for the ethyl alcohol as compared to a factor of 6.0 for the isopropyl alcohol. Hanzlik has pointed out that in general the more potent the digitalis medication the greater the margin of safety.

Our extract of digitalis with the official menstruum assayed 0.518 ± 0.054 cc. per cat unit which is equivalent to the active substance in 72.4 mg. of

powdered digitalis. Since the U. S. P. XII directs the use of 100 Gm. of crude drug to make 1000 cc. of tincture and since in preparing our extract, 100 Gm. (presumed to be of 68% potency) of crude drug were used to make a volume of 700 cc., which assays approximately 10/7 as potent as the official standard, we may conclude that our sample of powdered digitalis leaf was more nearly of 100% potency than 68%. Therefore our assay did not confirm the potency stated on the label. The fact that our extracts were much stronger than the stipulated U. S. P. strength, before bioassay, of liquid extracts of digitalis may help to explain the fact that the standard errors in our assays were slightly greater than the 5.7% allowed by the Pharmacopœia.

CONCLUSIONS

1. Two liquid extracts of *digitalis purpurea* have been prepared, the first, with the official ethyl alcohol–water menstruum and the second, with isopropyl alcohol substituted for ethyl alcohol.

2. Isopropyl alcohol dissolves 15% less extractive than ethyl alcohol and its solution yields 50% less total ash.

3. Bioassays by the pigeon emesis, the pigeon fatal dose and the U. S. P. XII cat methods disclose the isopropyl alcohol product to be, respectively, 28.5% more, 11.7% more, and 7.7% less potent than its ethyl alcohol counterpart. Thus, we may conclude that isopropyl alcohol as a menstruum produces an extract of equivalent or greater potency than that produced by the official menstruum.

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The Relative Toxicity of Ethyl and Isopropyl Alcohols as Determined by Long Term Rat Feeding and External Application*,†

By Lloyd L. Boughton

The literature on the pharmacology and relative toxicity of ethyl and isopropyl alcohols is extensive. The author has, however, found nothing to indicate the relative effects of these alcohols when administered orally and externally to litter-mate animals for periods longer than a few days or weeks.

The marked increase during recent years in the use of isopropyl alcohol as a solvent for external applications seemed to make this type of investigation desirable.

Grant (1, 2) has reviewed the published literature on the pharmacology of isopropyl alcohol prior to 1923. The Standard Alcohol Company (3) has reviewed much of the literature published since 1923.

EXPERIMENTAL

Three groups of male albino rats of unknown strain were used in this experiment. There were eight, eight and seven, respectively, in the two test and control groups. The rats were eight weeks old when the experiment was started. They were housed by groups in colony cages described in an earlier paper (4). Diet consisted of Purina Growena, a balanced chicken ration, fed dry to increase fluid consumption.

Ethyl and isopropyl alcohols were given to the test groups as 5% solutions in tap water. Controls were given tap water. The rats obtained the liquids from the ordinary type water bottle. Outlet tubes were of identical bore and shape. The animals were allowed all they would drink.

Fluid and Alcohol Consumption.—Alcohol administration was started November 1 and continued through July, a total of 304 days.

The controls consumed an average of 11.5 cc. of tap water per day per rat or 54 cc. per Kg. during the nine-month period.

Fluid consumption was considerably lower in both alcohol test groups. The ethyl alcohol group (8 rats) drank an average of 7.4 cc. of 5% solution per day per rat, or 41 cc. per Kg. The daily absolute ethyl alcohol consumption averaged 2.1 cc. per Kg. of rat, equivalent to 147 cc. for a 70-Kg. adult human being.

The isopropyl alcohol group took 6.8 cc. of 5% solution per rat per day or 37.4 cc. per Kg. of rat, or 1.87 cc. of absolute isopropyl alcohol per Kg. This is equivalent to 131 cc. per day for a 70-Kg. human being.

Mortality, General Welfare and Behavior.—All rats in the isopropyl alcohol group survived the experiment. One rat died in the control group on the 210th day, and one in the ethyl alcohol group on the 253rd day.

Unusual behavior effects were not observed in

* Presented to the Scientific Section, A. PH. A., Columbus meeting, 1943.

† We are indebted to the Cramer Chemical Co., Gardner, Kan., for the animals and materials used in this experiment.

either of the alcohol groups. Appetites seemed normal as compared to controls. Thirst was evidenced in both test groups by the fact that the animals gathered about the water bottles each time after filling but left without drinking. They never did become accustomed to the taste of the alcohol solutions.

All rats in both test groups developed noisy breathing after several months of alcohol administration. Forced activity increased the noise and apparent labor of breathing, none of which was observed in the controls. An earlier paper (4) has described a similar condition in aspirin-fed animals.

Weight Effects.—The 23 rats were divided into groups on a basis of weight and were weighed by groups every two weeks. Weight comparisons are shown in Table I. Weight depression was observed in both test groups after two weeks of feeding, being considerably more marked in the ethyl alcohol group than in the isopropyl alcohol group.

Weight depression was noticeably increased in both groups during the hot months of June and July when fluid consumption was increased also.

TABLE I.—WEIGHT COMPARISON IN GRAMS

	8 Wk.	16 Wk.	24 Wk.	36 Wk.
Controls	120	200	260	295
Ethyl	129 (+7.5%)	165 (−17.5%)	211 (−19.0%)	206 (−30%)
Isopropyl	125 (+4.0%)	173 (−13.5%)	223 (−14.0%)	226 (−23%)

Activity and Maze Learning.—All animals were placed on an elevated T-maze described in an earlier paper (5) for 15 trials after eight and one-half months of alcohol administration.

All of six controls made perfect runs during the 15 trial period. Three of seven in the ethyl alcohol group and four of eight in the isopropyl alcohol group failed to make perfect runs in 15 trials.

Six controls averaged 117 errors in 15 trials. Four ethyl alcohol animals averaged 127 errors, an increase of 8.5% over the controls. Four isopropyl rats averaged 136 errors, an increase of 16% over the controls. These comparison are significant only because of the fact that both test group rats made more errors than did the controls.

All alcohol-fed animals were extremely sluggish on the maze, as compared to controls. Little if any difference could be noted in the two test groups. Time comparisons, however, indicate a more marked sluggishness in the isopropyl alcohol group.

Six controls made nine errorless runs in 15 trials in an average time of 14.6 seconds. Three ethyl rats made one perfect run each in an average time of 17 seconds. Four isopropyl alcohol rats made six perfect runs in an average time of 21.5 seconds.

The controls averaged 19.3 minutes travel time as compared to 27 minutes (+40%) for ethyl alcohol rats and 37.5 minutes (+94%) for the isopropyl alcohol rats.

Total time comparisons for animals making errorless runs show similar variations. Controls averaged 111 minutes, ethyl rats 153 minutes (+38%) and isopropyl rats 169 minutes (+52%).

Withdrawal Effects.—After nine months of alcohol administration, the 5% alcohol solutions were replaced with tap water and the experiment continued for one month. The rats in the test groups gained weight rapidly during the 30-day withdrawal period, the isopropyl animals outweighing the controls. Average weights at the end of the period were as follows: controls 307 Gm., ethyl alcohol 289 Gm., isopropyl alcohol 317 Gm. Ethyl alcohol had a more permanent effect on weight depression than did isopropyl, or isopropyl alcohol had caused a greater dehydration.

The noisy breathing observed in both test groups during alcohol administration was almost completely eliminated during the month withdrawal period, suggesting that it may have been due to dehydration.

Fluid consumption was increased 37% in the ethyl alcohol group and 48% in the isopropyl alcohol group during the withdrawal period. This would seem to be ample evidence of dehydration in the test animals as a result of being forced to drink 5% solutions of the respective alcohols during the nine months.

All animals in all groups that had completed the maze in learning trials were returned to the maze for six trials after the month withdrawal period. The sluggishness observed in both test groups during the learning trials was much less marked during the relearning trials. Six controls made 11 perfect runs in an average time of 13.3 seconds. Three ethyl rats made four perfect runs in an average time of 14.8 seconds. Four isopropyl alcohol rats made seven perfect runs in an average time of 15.2 seconds.

Effect of External Application of Ethyl and Isopropyl Alcohols.—Five times each week during the nine-month period, ten drops of a 50% solution of the respective alcohol was placed on the head of each test animal with a medicine dropper. Marked irritation was evident with either alcohol at each application and the rats invariably carried the solution down over the face and into the eyes with the forepaws. The face area between the ears and eyes was saturated with each application. Controls were treated in the same manner with tap water.

These external applications were made 187 times during the experimental period. The hair, skin and eyes of each animal were observed carefully at each application and were watched carefully for a month after the applications ceased. At no time during the nine-month period or since has there been any evidence of injury to hair, skin or eyes that would differentiate the ethyl alcohol and isopropyl alcohol animals from the controls.

SUMMARY AND CONCLUSIONS

Five per cent solutions of ethyl and isopropyl alcohols have been given to groups of albino rats as their only water supply for nine months. Litter mate controls were given tap water.

Ethyl alcohol (absolute) was taken at an average daily rate of 2.1 cc. per Kg. of rat, equivalent to 147 cc. for a 70-Kg. human being.

Isopropyl alcohol (absolute) was taken at an average daily rate of 1.87 cc. per Kg., equivalent to 131 cc. for a 70-Kg. human being.

Both test groups suffered a marked reduction in fluid consumption as compared to controls, the ethyl alcohol group taking 36% less and the isopropyl alcohol group 41% less. Fluid consumption was increased, respectively, 37% and 48% in the test groups as compared to controls during a month withdrawal period following the test period.

Both test groups experienced rather

marked weight depression during the test period but gained rapidly on the controls after withdrawal. The isopropyl rats outweighed the controls after one month on tap water.

Noisy breathing was markedly evident in both test groups during the test period but disappeared almost completely during the withdrawal period.

Sluggishness on the maze was apparent in both test groups, being most marked in the isopropyl alcohol group. This sluggishness was only partially eliminated during the withdrawal period.

There was no significant difference in the death rate either in test or control groups during the experiment with the two alcohols.

One hundred eighty-seven daily applications of 50% solutions of the respective alcohols to the face areas of test animals failed to produce injury to skin, hair, or eyes that would differentiate the test animals from controls treated in a similar manner with tap water.

The author is of the opinion that dehydration played an important role in any variations noted between test and control animals. He concludes that when used under the conditions presented above isopropyl alcohol is only slightly more toxic than ethyl alcohol internally. Neither produces harmful effects externally.

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Use of Pectin in Pharmaceutical Pastes and Ointments*

By W. D. Maclay, Allan D. Shepherd and Harry Lotzkar

War and industrial hazards have accentuated the need for more effective means of preventing infection and of healing burns and wounds. Much attention has been given recently to the efficacy of various drugs and to the development of satisfactory washable gel- and emulsion-type bases in which to incorporate them. The physical characteristics of "ideal" products of this nature have been described in recent journal articles (1, 2).

When it became apparent that the war would deny the United States an adequate supply of the water-soluble vegetable gums normally imported from the Eastern Mediterranean countries, interest at once developed in substitues. At this laboratory pectin was investigated as a thickening agent to substitute for gum tragacanth in tannic acid pastes, with and without antiseptics, and as an emulsifying agent in the preparation of an ointment base, which would fulfill the requirements specified by the Division of Medical Sciences, National Research Council. A formula for each of these types, made available to the Laboratory of the AMERICAN PHARMACEUTICAL ASSOCIATION, has been included by Evans (2) in his survey of preparations for the treatment of burns.

In addition to properties which make it well adapted as a thickening and an emulsifying agent, pectin appears to possess certain therapeutic value. Of special interest is the work of Fantus and Dyniewicz (3) and Tompkins, *et al.* (4), on the use of pectin pastes in the treatment of bed sores and indolent ulcers. The National Formulary VII includes formulas for two pectin pastes.

The present paper is concerned with the preparation and the study of the stability and compatibility of (*a*) pastes prepared with pectin and gum tragacanth and (*b*) an ointment base stabilized with pectin. The stabilities of the pastes and base at −10° C., 22° C. and 55° C. were followed by means of measurements of viscosity and *p*H at regular intervals over a period of four months.

* Received July 3, 1943, from the Western Regional Research Laboratory, Albany, Calif., Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

MATERIALS AND METHODS

Tannic Acid Pastes.—A number of pastes containing tannic acid were prepared in various ways with different compositions and types of pectin.

The compositions of pastes which, of those prepared, most closely meet the specifications to which reference has previously been made are as follows:

	Per Cent
(*a*) Tannic acid paste:	
Tannic acid	10
Pectin (200-grade, high methoxyl, <100 p. p. m. iron)[1]	5
Glycerol	12
Sodium sulfite	0.2
Preservative (methyl *p*-hydroxybenzoate)	0.2
Ringer's solution (alkaline, to adjust *p*H of paste to 4 or above)	72.6

	Per Cent
(*b*) Tannic acid paste containing sulfa-drugs:	
Tannic acid	10
Pectin (200-grade, high methoxyl, <100 p. p. m. iron)	5
Glycerol	12
Sulfa-drugs (200 mesh or finer)	5
Sodium sulfite	0.2
Preservative (methyl *p*-hydroxybenzoate)	0.2
Ringer's solution (alkaline, to adjust *p*H of paste to 4 or above)	67.6

The tannic acid dissolved in the alkaline Ringer's solution at room temperature was added rapidly to a triturated mixture of pectin, sulfa-drug (when used), preservative, sodium sulfite and glycerol. The resultant mixture was triturated until smooth and uniform. Homogeneity of the product is improved by passing it through a hand homogenizer.

Ointment Base.—Of a number of ointments stabilized with pectin which were submitted to the National Research Council the one having the following composition was considered most satisfactory:

	Per Cent
Ringer's solution (alkaline, to adjust *p*H of ointment to 5)	57.8
Petrolatum (heavy liquid)	20
Glycerol	12
Sulfa-drug	5
Pectin (200-grade, high methoxyl)	5
Methyl *p*-hydroxybenzoate	0.2

Subsequently, stability studies were conducted on an ointment base having the following composition but varying in *p*H from 3 to 6:

	Per Cent
Ringer's solution (alkaline)	62.8
Petrolatum (heavy liquid)	20
Glycerol	12
Pectin (200-grade, high methoxyl)	5
Methyl *p*-hydroxybenzoate	0.2

The pectin was dispersed in the petrolatum in a jar to which the alkaline Ringer's solution containing the glycerol was added rapidly. The mixture was shaken until it was no longer fluid and then transferred to a mortar and triturated until smooth. The base was passed twice through a hand homogenizer, which not only improves the appearance of the product but also renders it more stable.

The method of preparation of an ointment may have to be modified, depending on the nature of the medicament. If the medicament is soluble in either water or oil the methods are essentially the same as described. If the medicament is insoluble in both, the following modification is recommended. Withhold the glycerol in the preparation of the base; disperse the medicament in the glycerol, and incorporate this mixture into the base by trituration.

Each paste and base was divided into three portions which were stored at −10° C., 22° C. and 55° C. Measurements of viscosity and *p*H were made at regular intervals. The Stormer viscometer and a glass electrode were used at a temperature of 22° C.

The compatibility of the paste and ointment base with various medicaments was observed for storage times extending from five months to a year.

RESULTS AND DISCUSSION

The results of this investigation will be discussed as they relate to the specifications (1) (2).

Washability.—The solubilities of all ingredients (exclusive of medicaments) incorporated into the paste are sufficient to make the applied product easily removable with water. The ointment base containing the oil in a highly dispersed state is likewise easily removable.

Distribution of Medicament.—A study of the distribution of medicaments of low solubility, *e. g.*, sulfanilamide, sulfadiazine and sulfathiazole, disclosed no significant settling of particles of 200 mesh or finer.

Color.—The tan to brown requirement of the tannic acid paste can be met if the pectin contains not more than 150 p. p. m. iron. Such pectin is commercially available.

*p*H.—The *p*H values of the paste and ointment base were adjusted by the use of alkaline Ringer's solution.

Consistency.—Pastes and ointments with good spreading characteristics are obtained when a concentration of about 5% of a 200-grade pectin with relatively high-methoxyl content is used. Low-methoxyl pectins which are sensitive to small amounts of calcium can be used provided the concentration of that metal is carefully controlled. Glycerol and propylene glycol have been used as conditioning agents and yield products of satisfactory consistency in concentrations of 10 to 20%.

General Stability.—(*a*) Temperature: The viscosities of the paste and ointment base stored at −10° C. showed no measurable changes even though they were brought back to room temperature several times. At 22° C. the paste experienced an increase in viscosity of about 10% after twenty weeks' storage and the viscosity of the ointment base increased about 25% over a storage time of four months. Pronounced changes in viscosity occurred on storage at 55° C. After one month the viscosity of both the paste and ointment base had fallen to about 10% and 25% of their initial values, respectively. Thereafter they exhibited a definite thickening until at the end of five months the products gelled. This phenomenon is due to slow demethoxylation of the pectin which changes its gelling characteristics. Analysis of the pectin recovered from the paste stored at 55° C. showed a decrease in methoxyl content of 25%. No significant demethoxylation occurred at the lower storage temperatures. Tragacanth pastes, prepared for comparison with the pectin pastes, suffered a loss in viscosity at 55° C. of the same order as that for the pectin paste.

Table I shows the effect of storage time and temperature on the viscosity of tragacanth and pectin pastes.

Tables II and III show the effect of storage time and temperature on the *p*H of the pastes and ointment base, respectively.

[1] N. F. VII pectinum was satisfactory but since iron tolerance has not been specified there may be considerable variations from one lot to another. However, low-iron pectin may easily be prepared by the method of Olsen (5) for the purification of pectin.

TABLE I.—EFFECT OF STORAGE TIME AND TEMPERATURE ON THE VISCOSITY OF TRAGACANTH AND PECTIN PASTES

Paste	Storage Temp., ° C.	Initial Viscosity Index	Viscosity Index After 4 Wk.	8 Wk.	12 Wk.	16 Wk.	20 Wk.
Tragacanth	22	40	45	50	60	60	60
Pectin	22	100	95	95	95	100	95
Tragacanth	55	40	20	6	4	4	4
Pectin	55	95	17	8	8	19	Gelled

TABLE II.—EFFECT OF STORAGE TIME AND TEMPERATURE ON THE *p*H OF THE PASTES

Paste	Storage Temp., ° C.	Initial *p*H	*p*H of Paste after 4 Wk.	8 Wk.	12 Wk.	16 Wk.	20 Wk.
Tragacanth	22	4.2	4.1	4.0	4.1	4.1	3.9
Pectin	22	4.2	4.0	4.0	4.2	4.1	3.9
Tragacanth	55	4.1	3.8	3.6	3.7	3.6	3.6
Pectin	55	4.2	3.2	3.4	3.5	3.3	Gelled

TABLE III.—EFFECT OF STORAGE TIME AND TEMPERATURE ON THE *p*H OF THE OINTMENT BASE

Storage Temp., ° C.	Initial *p*H of Base	*p*H after 4 Mo.	Storage Temp., ° C.	Initial *p*H of Base	*p*H after 4 Mo.
−10	3.2	3.2	−10	5.9	5.7
22	3.2	3.1	22	5.9	4.7
55	3.2	Gelled	55	5.9	3.3
−10	4.2	4.2	−10	6.2	5.7
22	4.2	4.0	22	6.2	4.8
55	4.2	3.0	55	6.2	3.3
−10	5.2	5.1			
22	5.2	4.6			
55	5.2	3.2			

Thus, at lower temperatures pectin pastes and ointments prepared with pectin are reasonably stable but deteriorate rapidly at elevated temperatures. Tragacanth pastes behave similarly at elevated temperatures.

(*b*) Mold and microörganism growth: Methyl *p*-hydroxybenzoate, 0.2%, was sufficient to prevent microbial growth. It was beyond the scope of the present investigation to study the antiseptic value of the preparations.

(*c*) Discoloration: Either of two factors may cause discoloration in tannic acid–pectin pastes. Tannic acid reacts with iron to produce a dark purple or almost black color. This can be avoided by the use of pectin of low-iron content and by avoiding contact with iron during the preparation of the paste. The other cause of discoloration may be oxidation of the tannic acid. The addition of 0.2% sodium sulfite as an antioxidant (6) prevented discoloration even at 55° C. when the materials were stored in the dark. In direct sunlight, however, color development is inhibited for short periods of time. On prolonged standing in a partially filled closed glass jar the surface begins to darken. Storage in collapsible, nonferrous metal tubes should eliminate discoloration from this cause. When stored at 55° C. the ointment base became discolored but at room temperature the discoloration was slight.

Medicated Pastes and Ointments.—Satisfactory pastes of the following medicaments and combinations of medicaments were prepared in the percentages noted:

Gentian violet 1%
Acriflavine hydrochloride 1%
Salicylic acid 3%
Sulfur 10% and 15%
Sulfathiazole 5%
Picric acid 1%
Tyrocidine 50 γ/Gm.
Sulfanilamide 5%
Brilliant green 1%
Coal tar 5%
Phenol 2%
Oil of birch tar 10%
Tyrothricin 50 γ/Gm.
Gramicidin 50 γ/Gm.
Acriflavine hydrochloride 1%, brilliant green 1%, gentian violet 1%
Salicylic acid 3%, benzoic acid 6%
Salicylic acid 2%, sulfur 5%
Tannic acid 10%, phenol 0.5%
Tannic acid 20%, acriflavine hydrochloride 0.1%
Tannic acid 2.5%, mercuric chloride 0.1%
Tannic acid 10%, salicylic acid 0.1%
Resorcinol 2%, coal tar 5%

Incompatibilities resulted in the following pastes:

Silver nitrate 5%
Tannic acid 5%, silver nitrate 10%
Gentian violet 1%, silver nitrate 5%
Coal tar 10%, zinc oxide 10%
Zinc carbonate 8%

Ointments containing the following medicaments were prepared. The composition in liquid petrolatum, glycerol and pectin was kept constant at 20%, 12% and 5%, respectively.

Sulfanilamide 5%
Sulfathiazole 5%
Benzoyl peroxide 5%
Allantoin 10%
Zinc oxide 10%
Coal tar 5%
Birch tar 10%
Ammoniated mercury 10%
Tannic acid 10%, sodium sulfite 0.2%

Tannic acid 10%, sulfadiazine 5%, sodium sulfite 0.2%
Tannic acid 10%, sulfanilamide 5%, sodium sulfite 0.2%
Salicylic acid 3%, benzoic acid 6%
Sulfanilamide 5%, urea 20%
Sulfadiazine 5%
Sulfur 10%
Phenol 2%
Burow's solution 10%
Zinc carbonate 10%
Ichthammol 5%
Balsam peru 10%

As in the case of the medicated pastes, ointments containing ions of heavy metals were incompatible. In addition the ointments containing sulfur, ichthammol and birch tar were incompatible.

SUMMARY

1. Tannic acid–pectin paste and an ointment base stabilized with pectin were prepared and a study was made of their general stability over a period of four months.

2. Various medicaments were incorporated into the paste and base, and some incompatibilities were observed.

3. Freezing arrested changes in the paste and base. At room temperature the pastes and bases gradually became more acidic but were stable. At 55° C. they became discolored and gelled after five months' storage.

4. The paste and base fulfill the requirements specified by the National Research Council except when stored at 55° C.

5. A comparison between the pectin paste and the tragacanth paste indicated that pectin and tragacanth have the same advantages and disadvantages.

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A Rapid and Simple Method for the Determination of Ephedrine*

By Karl Schoen

The quantitative assay of ephedrine and its salts in pharmaceutical preparations presents various difficulties inherent in the great solubility of the alkaloid in water, its low melting point and its high volatility. The official assay methods (1, 2, 3) are not entirely satisfactory because they give either inconsistent results or are too laborious and time consuming. These difficulties are reflected in the various official methods for assay of the drug. Each type of preparation containing the drug requires special methods of treatment before the alkaloid is actually determined. Such additional steps introduce the possibility of error.

The determination of ephedrine in Ephedrine Spray and Compound Spray of Ephedrine according to N. F. VI and VII is especially annoying, since a preliminary extraction of the alkaloid from the mineral oil is necessary. This may be a source of serious error. The assay of ephedrine in the presence of other alkaloids like epinephrine is still more complicated. The simple polarimetric assay cannot be used with accuracy in the case of ephedrine, because the optical rotation of the base varies within wide limits with the concentration and nature of the solvent, and cannot be employed when the racemic base is present.

The present paper describes a new method for the quantitative assay of ephedrine and its salts, which is simple, rapid and accurate. It can be used for all the common ephedrine preparations even in the presence of certain other alkaloids.

The new method is based on the observation that ephedrine is steam distilled quantitatively in the presence of an excess of strong alkali. The ephedrine can be directly determined by acidimetric titration of the distillate. The steam distillation is conveniently performed in a Kjeldahl distillation apparatus.[1] The distillate is collected in an excess of volumetric acid, which is then back titrated in the presence of methyl red, exactly as in a Kjeldahl determination.

If the determination is properly conducted, it gives accurate and reliable results. We have made routine determinations on as

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[1] We have used, with advantage, the Micro-Kjeldahl apparatus, with ground joints, manufactured by Scientific Glass Co., Bloomfield, N. J., No. M-3065, equipped with 100-ml. flasks.

much as 400 mg., and as little as 20 mg. with the same accuracy and speed. A determination of 10 mg. of ephedrine hydrochloride, corresponding to 8.1 mg. of free ephedrine or 0.6 mg. of nitrogen, has been made with less than 1% error. The average error of the method appears to be less than 0.5% The new assay method permits the performance of three assays in one hour, whereas the usual extraction methods require about two hours for each determination.

It has been found that under the conditions of the method the ephedrine distills without decomposition, and even racemization of optically active ephedrine does not occur. Thus, in a qualitative identification of the distillate from l-Ephedrine Sulfate, we have extracted the alkaline distillate with chloroform according to the directions of U. S. P. XII. After twenty-four hours, upon spontaneous evaporation of the chloroform solution, l-Ephedrine Hydrochloride can be isolated in pure state. A small quantity of benzaldehyde and an aliphatic amine, which could be recognized by their characteristic odor, seem to have been formed as secondary products in the chloroform solution rather than during the actual distillation.

The same assay method can be used for the quantitative determination in pharmaceutical preparations of amphetamine (1-phenyl-2-amino-propane), which is also volatile with steam. As amphetamine is much less soluble in water than ephedrine, the determination is much faster in this case.

Epinephrine, which has a structure similar to ephedrine, does not steam distill, nor is it cleaved to form a volatile base under the conditions of the assay. Only when heated with 50% NaOH to 180–200° is epinephrine split chemically to the extent of about 25% in half an hour.

A similar assay method as that described here is advised in the official methods of the Association of Agricultural Chemists for procaine (4). While we have had good results for this base, both alone and in the presence of epinephrine, we have found that in order to obtain quantitative results, the distillation must be continued for about one-half hour and at least 150–200 ml. distillate must be collected. In the case of procaine, the end point of the titration is not so sharp as with ephedrine.

TABLE I

Preparation	Assay Method	Anhydrous Theory	Ephedrine Found	Per Cent
Ampuls ephedrine sulfate, 3/4 Gr. per 1 cc. (analytical No. 6801)	U. S. P. XI, 2nd Suppl.	0.576 Gr.	0.575 Gr.	99.8
	New method	0.576 Gr.	0.573 Gr.	99.5
Ephedrine inhalant plain (analytical No. 3036-F), content/1 Gm.	N. F. VI	10.00 mg. (labeled)	10.77 mg.	
			10.52 mg.	
		Av.	10.64 mg.	
	New method	.....	10.65 mg.	
			10.64 mg.	
Rac. ephedrine hydrochloride powder (analytical No. 557-P), 1.0000 Gm. dissolved in 100 ml. water				
5-ml. samples (50 mg. Ephedrine HCl)	New method	81.91%	80.95%	98.8
			81.28%	99.2
1-ml. samples (10 mg. Ephedrine HCl)	New method	81.91%	81.90%	99.9
			81.90%	99.9
Ephedrine ointment (analytical No. 5014) by weight	New method	.....	1.15%	
			1.16%	
			1.17%	
			1.16%	
Compound spray of ephedrine, N. F. VI by vol.	New method	.....	0.80%	
			0.81%	
			0.83%	
			0.83%	

TABLE II.—AMPHETAMINE (COMMERCIAL SOLVENTS CORPORATION D-100)

Sample Weight, Gm.	Found, Gm.	Per Cent
0.1437	0.1440	100.2
0.1409	0.1392	98.8
0.1314	0.1305	99.3
0.0105	0.0107	101.9
0.0105	0.0107	101.9
0.0105	0.0106	101.0

EXPERIMENTAL

Assay of Ampuls Ephedrine Hydrochloride or Ephedrine Sulfate.—An aliquot of the ampul solution containing between 20 and 200 ml. of ephedrine is pipetted into the digestion flask of a Micro-Kjeldahl apparatus; the flask is connected with the apparatus and 10 to 15 ml. of 50% NaOH is added. The mixture is then distilled with a rapid

current of steam, and the distillate collected in an excess of standard volumetric sulfuric acid. About 50–150 ml. of distillate is collected according to the amount of ephedrine present. The end of the distillation is indicated by the neutral reaction of the distillate to litmus paper. The excess acid is back titrated with standard sodium hydroxide solution, using methyl red as indicator and titrating to a salmon-pink end point. As ephedrine is a strong base, the end point is very sharp. It has been found very convenient to use only a slight excess of acid, and to back titrate with $N/50$ alkali using a microburette calibrated in 0.02 ml. with a capacity of 5 ml.

1 ml. of $N/10$ H_2SO_4 = 0.01652 Gm. anhydrous ephedrine

Assay of Solution of Ephedrine Sulfate, Ephedrine Spray, Compound Spray of Ephedrine and Syrup Ephedrine Sulfate.—A volume of the solution containing between 20 and 200 mg. of ephedrine is pipetted into the Kjeldahl digestion flask, and after the addition of 10–15 cc. of NaOH the assay is executed as described above. In the case of Ephedrine Spray and of Compound Spray of Ephedrine a little mineral oil or aromatic substances are carried over with the steam. This floats on the surface of the distillate, but does not interfere with the titration.

Assay of Tablets Ephedrine Hydrochloride and Jelly of Ephedrine Sulfate.—A representative sample of the tablets or jelly is weighed in a small beaker, cut from an ampul, and the beaker is transferred to the Kjeldahl flask. Fifty per cent NaOH is added and the distillation carried out as described under ampuls Ephedrine Hydrochloride.

Assay of Amphetamine.—The sample (20–200 mg.) is weighed off in a tared, open ampul and transferred to the Kjeldahl flask. The distillation is carried through exactly as in the case of ephedrine. About 40–50 cc. of distillate must be collected in order to complete the distillation.

1 ml. of $N/10$ H_2SO_4 = 0.01351 Gm. amphetamine

Identification of Ephedrine in the Distillate.—Two hundred milligrams l-Ephedrine Sulfate, U. S. P. XII, was steam distilled from strongly alkaline solution exactly as described above. The distillate was made alkaline with sodium hydroxide solution and extracted with 4–15-ml. portions of chloroform. The chloroform solution was filtered through cotton and stored in a stoppered flask for twenty-four hours, according to the directions of the U. S. P. XII, p. 162.

After the spontaneous evaporation of the chloroform, crystals, contaminated with a little oil, had separated. The odor of the oil indicated the presence of benzaldehyde and an aliphatic amine. It was removed by washing with a small portion of chloroform, and the residue recrystallized from a mixture of absolute ethanol and ether; m. p. 219–220°.

M. p. of pure l-Ephedrine hydrochloride (Merck, U. S. P.)	220–221°
M. p. of rac. Ephedrine HCl	187–188°
Mixed m. p., with l-Ephedrine HCl	219–220°

Table I contains representative examples comparing the new method with the official methods of assay recommended for the specific preparations.

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(1) "Pharmacopœia of the United States," Twelfth Revision, Mack Printing Company, Easton, Pa.,1942, p. 164.

(2) "The National Formulary," Seventh ed., Mack Printing Company, Easton, Pa., 1942, pp. 41, 201, 293, 294.

(3) "Official and Tentative Methods of Analysis," Fifth ed., Association of Agricultural Chemists, Washington, D. C., 1940, p. 585.

(4) *Ibid.*, p. 591.

A Study of the Preparation of Mercurial Ointment*

By N. A. Kessler† and H. G. DeKay‡

Mercurial ointment has been used in medicine for at least a thousand years, and today it still has an important place in materia medica. The preparation of this ointment, due to its difficulty, has been a challenge to pharmacists from the first, and as a result numerous formulas have been proposed, each of which represents an attempt to shorten the tedious operation of dispersing the mercury. Even at the present time, the preparation of mercurial ointment is a problem. In fact, it is considered so difficult that the pharmacist would not think of making it.

The object of this study was to investigate the methods of preparing mercurial ointment and attempt to improve them or devise new methods whereby the ointment could be made in less time and with less trouble.

Mercurial ointment may be prepared by two methods: the dispersion method and the precipitation method. In the former, the mercury is subdivided and dispersed in an ointment base by agitation of some sort. In the precipitation method, a mercury compound is reduced to free mercury by a suitable reducing agent, and the particles of mercury thus obtained incorporated in an ointment base.

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The preparation of mercurial ointment by the dispersion method is analogous to making an emulsion. An emulsifying agent is necessary in order to facilitate the dispersion of the mercury. Such an agent must be capable of forming a protective film around the globules of mercury as fast as they are formed by the agitation, thus preventing their coalescing. The use of the term emulsifying agent in this paper will refer to this property.

Substances that have been used to disperse mercury for preparing the ointment may be put into three classes: (*a*) fatty substance such as lard, suet, petrolatum, wool fat, oleate of mercury, vegetable oils, etc.; (*b*) nonfatty substances such as plant extracts, tinctures, resins, etc.; and (*c*) mixtures of fatty and nonfatty substances.

The work of Culbertson and Hedman (1) and Culbertson and Luedtke (2) show that, in order for the interfacial tension between mercury and oil to be lowered, certain substances contained in the oil must be adsorbed at the interface.

Their experiments indicate why certain substances are capable of emulsifying mercury and others are not. Those substances containing constituents capable of being adsorbed at the interface of the mercury globules and the substances will act as emulsifiers, since the film formed by the adsorption will prevent coalescence of the mercury globules. Those substances not containing constituents capable of being adsorbed at the interface will not act as emulsifiers. Just how efficient an emulsifier a certain substance is depends upon how strongly its constituents are adsorbed.

The precipitation method, although not new, has come into prominence in the last few years. In 1845, Orosi (3) presented a method of making mercurial ointment whereby a solution of mercury bichloride was reduced with an excess of stannous chloride solution containing hydrochloric acid. The precipitated mercury obtained by the reduction was washed, dried between bibulous paper, and mixed with fresh lard.

Dieterich (4) mentions a method of making the ointment in which sulfurous acid gas was passed into a solution of mercuric nitrate, and the precipitate obtained incorporated in a fatty base.

Archetti (5) proposed a formula for preparing mercurial ointment in which the mercury was obtained by reducing mercury bichloride with hydrogen peroxide in the presence of sodium hydroxide.

Polacci (Conci, 6) reduced mercury bichloride with glucose solution in the presence of potassium hydroxide and then incorporated the precipitate, after washing, in sufficient fat.

In 1927, Jordan and Brody (7) presented a method of preparing mercurial ointment by the reduction of mercury bichloride with formaldehyde in the presence of gelatin. In 1932, Jordan *et al.* (8), presented an improvement of this formula.

Holdermann (9) also used mercury bichloride and formaldehyde in making mercurial ointment. However, the reduction was carried out in the presence of heavy vaseline oil and methanol instead of water. Later in the same year (10), he proposed a modification of his formula.

EXPERIMENTAL

I. Dispersion Method; Emulsifying Power of Oils and Fatty Substances.—To test the ability of various fixed oils, petrolatum white petrolatum, fresh lard, rancid lard, oleate of mercury and wool fat, to emulsify mercury, portions of the metal were triturated in a mortar with one-third their weight of these substances. The results are shown below in Table I.

TABLE I

	Oil or Fat Used	Result
1.	Mineral oil	Mercury globules readily coalesced
2.	Sweet almond oil	Mercury dispersed in large globules
3.	Linseed oil	Mercury dispersed in large globules
4.	Olive oil	Mercury dispersed in large globules
5.	Mineral oil and white petrolatum	Poor dispersion even after prolonged trituration
6.	Linseed oil and white petrolatum	Mercury dispersed quite rapidly
7.	Olive oil and white petrolatum	Mercury dispersed quite rapidly
8.	Petrolatum	Poor dispersion even after prolonged trituration
9.	White petrolatum	Poor dispersion even after prolonged trituration
10.	Fresh lard	Poor dispersion even after prolonged trituration
11.	Rancid lard (1 year old)	Mercury dispersed rapidly but not completely
12.	Rancid lard and white petrolatum	Mercury dispersed rapidly and completely
13.	Wool fat	Mercury dispersed rapidly and completely
14.	Oleate of mercury	Mercury dispersed after prolonged trituration

From the above table it can be seen that such substances as sweet almond oil, linseed oil and olive oil are not valuable as emulsifiers until their viscosity has been increased.

In this experiment it was observed that the substances which were good emulsifiers of mercury were wetted by the metal; that is, the mercury tended to spread out and adhere strongly to the substance.

Emulsifying Power of Water Soluble Substances.—It is a common demonstration in colloidal chemistry to show how readily mercury can be subdivided by shaking with a solution of tannic acid.

In order to see if other colloid-forming substances, behaved in a similar manner, mercury was shaken with aqueous solutions of acacia, tragacanth, egg albumen, and soap. The results were the same as those obtained with tannic acid. It was also found that a small amount of bichloride of mercury increased the emulsifying power of most of these colloid-forming substances, especially tannic acid.

Application of Dispersion Agents to the Preparation of Mercurial Ointment; Oleate of Mercury.—Three samples of stronger mercurial ointment were prepared by the U. S. P. XI method, using a different brand of mercury oleate for each. In preparing the first ointment, fifteen minutes were required to disperse the mercury. In preparing the second and third samples, about thirty minutes were required for each.

The above experiments show two things: (1) Even under the most favorable conditions, the preparation of mercurial ointment by the U. S. P. method requires considerable laborious trituration. (2) There is a variation in different brands of U. S. P. oleate of mercury which influences the time required to disperse mercury with them.

Wool Fat.—Twenty-five-gram samples of mercury were triturated with 10-g. portions of wool fat at temperatures ranging from 25° C. to about 45° C. At 30° C. the consistency of the wool fat was just right, and the mercury was dispersed in less than ten minutes. At 25° C. the mercury was dispersed in ten minutes, and at 45° C. (wool fat melted), at least fifteen minutes of trituration was required to disperse the metal.

Tannic Acid and Mercury Bichloride Mixture.—Of the water-soluble substances capable of emulsifying mercury, tannic acid and soft soap were considered the best. They are easier to manipulate and are less likely to spoil than are acacia, tragacanth, gelatin and egg albumen.

Since the viscosity of an aqueous solution of tannic acid and mercury bichloride is too low to permit satisfactory dispersion by trituration, a paste was used. Experiments showed that a paste consisting of 1 Gm. each of tannic acid and mercury bichloride was the most efficiency for dispersing 50 gm. of mercury.

During the course of the above experiments, there was great difficulty in keeping the tannic acid-bichloride paste at the proper consistency for best dispersion. This was finally overcome by using glycerin instead of water, and then incorporating the mixture in a portion of wool fat before adding the mercury. The mixture thus produced was capable of dispersing mercury in about one-third the time required for plain wool fat at the same temperature.

Utilizing the rapid dispersion property afforded by this combination, the following formula was devised for preparing stronger mercurial ointment:

FORMULA 1

Mercury	50 Gm.
Tannic Acid	1 Gm.
Mercury Bichloride	1 Gm.
Glycerin	4 Gm.
Wool Fat	30 Gm.
White Wax	5 Gm.
White Petrolatum	9 Gm.
To make	100 Gm.

Triturate the tannic acid, the mercury bichloride (previously powdered) and the glycerin together until a uniform mixture is obtained. To this add about one-half the wool fat and mix throughly. Add the mercury and triturate until dispersed, continuing the trituration for about five minutes after the globules have become invisible to the naked eye. Melt the remainder of the wool fat, the white wax and the white petrolatum over a water bath, allow to cool and add to the above mixture. Mix until a uniform ointment is obtained.

Ointments prepared according to this formula failed to show the presence of any free mercury bichloride when tested with stannous chloride and potassium iodide.

Soft Soap and Mercury Bichloride Mixture.—Previous experiments have shown that an aqueous solution of soft soap is a good emulsifier of mercury. However, since the viscosity of the aqueous solution is too low for rapid dispersion by trituration, a paste was used. Such a paste, consisting of 5 Gm. of soap and sufficient water, was capable of dispersing 50 Gm. of mercury, but not so well as the tannic acid-bichloride combination.

The next experiment involved the use of mercury bichloride in conjunction with soft soap. One gram of mercury bichloride was triturated with 5 Gm. of soft soap forming an orange-yellow paste. This paste showed great ability to disperse mercury, but it became too stiff as the dispersion proceeded. This was remedied by incorporating the paste in 20 Gm. of wool fat before adding the mercury.

Further experiments were carried out to determine what proportion of soft soap and mercury bichloride resulted in the bext mixture. The most satisfactory mixture for preparing 100 Gm. of 50% ointment was found to consist of 1 Gm. of mercury bichloride and 3 Gm. of soft soap.

Using the data obtained from these experiments, the following formula was devised for the preparation of stronger mercurial ointment:

FORMULA 2

Mercury	50 Gm.
Soft Soap	3 Gm.
Mercury Bichloride	1 Gm.
Wool Fat	30 Gm.
White Wax	5 Gm.
White Petrolatum	11 Gm.
To make	100 Gm.

Melt the wool fat, white wax, and white petrolatum on a water bath. Triturate the soft soap with the mercury bichloride (in a fine powder) thoroughly, and immediately add about one-third of the melted base. Mix well. To this mixture add the mercury and tritutate until dispersed, continuing the trituration for five minutes after the globules have become invisible to the naked eye. Finally add the remainder of the base, which has cooled, and mix until a uniform ointment is obtained.

Ointments prepared according to this formula failed to show the presence of any free mercury bichloride when tested with stannous chloride and potassium iodide.

Table II shows a comparison between ointments made by Formulas 1 and 2.

II. Precipitation Method.—The precipitation method of preparing stronger mercurial ointment presented by Jordan, *et al.* (8), was studied from a standpoint of mercury compounds used as sources of mercury, alkalies, protective colloids, reducing agents and methods of purifying the precipitated mercury as follows:

1. Mercuric nitrate and yellow mercuric oxide were substituted for mercury bichloride in the formula. The oxide gave good results while there

was no apparent difference in the use of the nitrate.

2. Stronger ammonia water was substituted for the sodium hydroxide with unsatisfactory results.

3. Powdered gelatin, powdered acacia, egg albumen, tannic acid, soft soap, hard soap, oleate of mercury and glycerin were substituted for leaf gelatin. None of them were found to have any advantage over leaf gelatin. The precipitate formed in the presence of the soap and oleate of mercury, although not so fine, was more miscible with the base than that produced with gelatin. A combination of gelatin and mercury oleate produced excellent results.

4. Hydrogen peroxide solutions, both 3% and 30%, were substituted in the formula for the formaldehyde solution. They did not produce complete reduction and the gas given off results in troublesome foaming.

5. In the Jordan, *et al.* (8) method, the precipitated mercury is washed with water to remove undesirable products of the reaction. This was observed to be unsatisfactory for two reasons: (1) The precipitate which settles rapidly after reduction settles more slowly as additional water is added. This slows down the process considerably. (2) Due to the fact that the precipitate is readily dispersed in water, there is danger of losing portions of it if the decantation is not carried out with the utmost care.

TABLE II

Formula	Consistency	Average Size of Particles[a]	Approximate Time Required to Disperse the Mercury	Percentage of Mercury
1	Good	20μ	3 min.	49.03
2	Good	20μ	1½ min.	50.50

[a]The size of the particles was measured with an optical micrometer.

To eliminate these faults, if possible, experiments were performed in which the precipitate was freed of impurities by (*a*) dialyzing, (*b*) washing the precipitate with water after it had been incorporated in a portion of the base, (*c*) washing the precipitate with alcohol. The first two of these failed to alleviate the objections to the previous procedures. The third method of washing the precipitate with alcohol proved very satisfactory, the precipitate settling very rapidly after each portion of alcohol, thus permitting rapid and easy washing. The remaining alcohol was removed by mixing the precipitate with a portion of the base and then heating over a water bath.

The only difficulty encountered with alcohol was that it tended to make the precipitate slightly lumpy. This was overcome by adding a few cc. of distilled water to the washed precipitate before incorporating the base. Upon heating this mixture to remove the alcohol, the lumps of precipitate were broken down.

As a result of the above studies of the precipitation method, the following formulas are suggested for preparing stronger mercurial ointment. These formulas are modifications of the process presented by Jordan, *et al.*, in 1932 (8).

FORMULA 1

Yellow Mercuric Oxide	56 Gm.
Sodium Hydroxide	15 Gm.
Gelatin (leaf)	4 Gm.
Solution of Formaldehyde	40 cc.
Wool Fat	30 Gm.
White Wax	5 Gm.
White Petrolatum, *q. s. ad*	100 Gm.

This process differs chiefly from the Jordan, *et al.* (8), process (1932) in that (*a*) yellow mercuric oxide is used as a source of mercury instead of mercury bichloride, and (*b*) the precipitated mercury is washed with alcohol instead of water. These changes result in more rapid preparation of the ointment and less danger of loss of mercury during the washing.

FORMULA 2

Yellow Mercuric Oxide	56 Gm.
Sodium Hydroxide	15 Gm.
Gelatin (leaf)	2 Gm.
Oleate of Mercury	2 Gm.
Solution of Formaldehyde	40 cc.
Wool Fat	30 Gm.
White Wax	5 Gm.
White Petrolatum, *q. s. ad*	100 Gm.

This formula differs from Formula 1 in that oleate of mercury is used in conjunction with gelatin as a protective colloid. This substance tends to make the precipitate more miscible with the base.

Table III shows a comparison between ointments made by Formulas 1 and 2.

TABLE III

Formula	Consistency	Average Size of Particles[a]	Approximate Time Required to Disperse the Mercury	Percentage of Mercury
1	Good	3μ	30 min.	49.50 50.79
2	Good	3μ	30 min.	51.03 50.46

[a]The sizes of the particles were measured with an optical micrometer.

SUMMARY AND CONCLUSIONS

1. The time required for preparing mercurial ointment by the dispersion method has been considerably shortened. Formulas for the improved method are presented.

2. The precipitation method of preparing mercurial ointment has been simplified and improved. Formulas incorporating the improvements are presented.

3. Sweet almond oil, linseed oil, olive oil, rancid lard, wool fat, and aqueous solutions of tannic acid, acacia, tragacanth, egg albumen, gelatin and soap were found to be capable of dispersing mercury.

4. Wool fat is a better dispersing agent for mercury than oleate of mercury.

5. A mixture of tannic acid and mercury bichloride in glycerin and a mixture of soft soap and mercury bichloride are excellent for dispersing metallic mercury in the presence of a fat.

6. The following conditions are necessary for the rapid dispersion of mercury by trituration:

(*a*) A protective film must be formed around each globule of mercury;
(*b*) The dispersing mixture must be the correct consistency or viscosity;
(*c*) The dispersing mixture must be wetted by the mercury.

7. The substitution of yellow mercuric oxide for mercury bichloride in the precipitation method of preparing stronger mercurial ointment shortens the time of preparation without interfering with the qualities of the ointment.

8. Washing the precipitated mercury (precipitation process) with alcohol shortens the time of preparation and diminishes the chance of losing mercury by decantation.

9. Mercury oleate used in conjunction with gelatin in the precipitation process renders the precipitated mercury more miscible with the base.

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Use of the Killifish, *Fundulus heteroclitus*, in the Assay of Marihuana*,†

By H. E. Warmke

In previous communications to THIS JOURNAL other workers (1, 2, 3, 4) have reported various studies on the activity of hemp, *Cannabis sativa*. These include the influence of variety, age, climatic conditions, etc., on the activity, and suggest the desirability of breeding programs aimed at the production of strains of fiber hemp with reduced potency. With plantings of hemp increased a hundredfold in this country, owing to the war and resulting interruption of fiber shipments from abroad, the need for such strains has greatly increased.

The chief obstacle to breeding experiments has been the lack of a simple and reliable method of determining the active resin content of individual plants. The Beam tests, which are chemical methods of assay, are of doubtful value. It is known that cannabidiol is responsible for the Alkaline Beam test and is physiologically inactive, whereas tetrahydrocannabinol, the known physiologically active component of the resin, is inactive to the Beam test. The standard biological assay method, utilizing the dog, is obviously not practical for the analysis of hundreds of individual plants required by a selection and breeding program.

Robinson (4) suggested a simpler biological assay in which goldfish were used as test animals. As a measure of the active principles he used the survival time of goldfish placed in a liter of water containing 2 cc. of an acetone extract of dry hemp leaves. His studies indicate: (*a*) that extracts of leaves of different hemp varieties are differently toxic to goldfish; (*b*) that the region in which the plants are grown and their age at the time of testing affect the degree of toxicity to goldfish; (*c*) that fractions of purified resins act in a somewhat similar manner upon dogs and fish; and (*d*) that different varieties of hemp have the same relative toxicity to fish and to dogs. This suggested the usefulness of fish in assaying marihuana for breeding tests, and led to our experiments.

It is the purpose of the present paper to report certain modifications of the Robinson method and to give preliminary results obtained with the modified method in actual breeding work. The hemp variety "Kentucky," furnished by the U. S. Department of Agriculture, Division of Cotton and Other Fiber Crops and Diseases, was used for all tests except in the polyploid series, where

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† Coöperative project with Division of Cotton and Other Fiber Crops and Diseases, U. S. Department of Agriculture.

seed obtained from commercial bird feed was used.

EXPERIMENTAL

The Test Animal.—The location of this laboratory on a harbor of Long Island Sound prompted the use of the common killifish, *Fundulus heteroclitus*, as a test animal, instead of the goldfish. The killifish may be taken from the harbor in large numbers at any time from early spring to late fall. An unlimited supply of fish that can be collected daily and used immediately in tests is an obvious advantage.

In addition to being readily available, the killifish is extremely hardy. Two young animals of the sort used in the tests (3.0–3.5 cm. in length and approximately 500 mg. in weight) will live for a week or longer in a half-pint milk bottle containing 100 cc. of harbor water. This is well beyond the duration of the tests, which usually run for only 24 hours. The use of quantities of water as small as 100 cc. in tests has important advantages: it permits the numerous tests to be run in a minimum of space in the constant-temperature room, and it allows the use of much smaller leaf samples.

Some difficulty was experienced late in the season when a few young of a related species, *Fundulus majalis*, were collected along with *F. heteroclitus*, and the mixed animals unwittingly used in tests. *F. majalis*, the striped killifish, is much less hardy than the common killifish and frequently will not survive 24 hours under laboratory conditions. This led to considerable irregularity in results until the source of the trouble was found. It is reasonably easy to separate the 2 species, however, even at this age; and after the young of *majalis* were rejected no more difficulty was experienced.

Measuring Relative Toxicities.—Robinson used survival time at 28° C. as a measure of toxicity, a strong extract being expected to kill the fish in a shorter period of time than a weaker one. He did not use graded doses of the same extract to determine toxicity, but suggested the desirability of doing so.

The method reported here uses the minimum concentration required to kill fish in 24 hours at a temperature of 15° C. as a measure of toxicity. Four half-pint bottles, each containing two fish and 100 cc. of harbor water, are used to test each plant. To the first bottle 0.4 cc. of a particular extract is added, to the second 0.2 cc., to the third 0.1 cc. and to the fourth 0.05 cc., giving concentrations of 0.4, 0.2, 0.1 and 0.05%, respectively. This range of concentrations was chosen because it covers the potencies of most plants; however, a few plants were found which ran off this scale, and for this reason an extra concentration may be added when next season's tests are run.

It has been found convenient to rate plants according to the number of fish killed by their respective extracts, the limits being 0 and 8 (4 bottles with 2 fish each). Thus, the extract from a plant with relatively low activity might kill only 2 fish (0.4% concentration), while the extract from a plant with moderately high content might kill 6 fish (all animals through the 0.4, 0.2 and 0.1% concentrations). In the first case the minimum lethal concentration is 0.4%; in the second it is 0.1%. Similar series containing harbor water only, or corresponding amounts of acetone or acetone extracts of spinach and other nontoxic leaves, were used for controls. Deaths occurred infrequently in such controls, and when they did occur they were scattered.

There is some question as to how plants that kill an odd number of fish should be scored. The possibility of a threshold concentration killing 1 of 2 fish in a bottle and allowing the other to live was not anticipated when the method was planned, but this frequently occurs. In such cases it has been decided to give equal rank to odd and even numbers. This procedure seems justified by the fact that retests run on the same extracts tend to repeat the same odd number of fish killed, and also by the fact that there is no tendency for odd-numbered classes to be deficient in frequency distributions (Figs. 3 and 4).

Methods of Sampling and Preparing Extracts.—It was discovered after preliminary tests that a definite gradient in toxicity exists among the leaves of a single plant, those leaves near the base of the plant being low and those near the top high in marihuana activity. These results are presented later in some detail (Fig. 1). It was difficult, therefore, to obtain a reliable sample by removing leaves at a single level; rather, leaves from the entire height of the plant should be taken. Such sampling was accomplished without serious injury to the plant by removing the center leaflet from each leaf above the fourth node, the entire mass of leaflets so obtained serving as the sample. (Leaflets were not collected below the fourth node because of the tendency of the lower leaves to drop irregularly during the course of the season.)

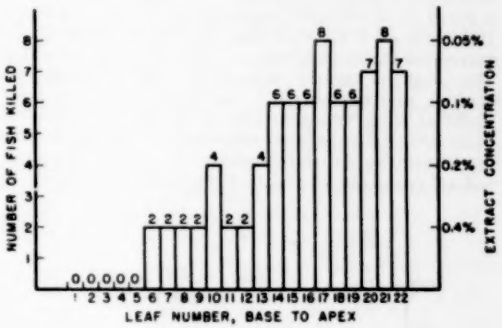

Fig. 1.—The relation of leaf position to active resin content in a single mature hemp plant.

Leaves are recorded in serial order on the horizontal scale, from number 1 at the base of the plant to number 22 at the apex. On the vertical scale, marihuana potency is plotted in terms of number of fish killed and of extract concentration. There is a sharp increase in activity of the leaves from base to apex of the plant.

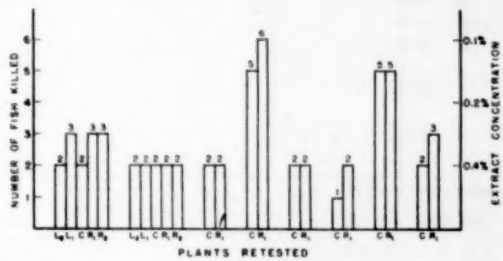

Fig. 2.—Results of successive marihuana-drug determinations on eight hemp plants.

***C* indicates center leaflet; R_1, L_1 indicate first leaflet to right or left, respectively, of center, etc. Samples from any one plant were taken on the same day. In no case do the results of retests differ by more than one fish from those of the initial test.**

This method of sampling has the important additional advantage that it allows for retesting of a plant by simply removing a second leaflet from each leaf. Retests have been made frequently throughout the course of this work to check the reliability of the methods.

Leaf samples are collected in small paper bags, and the open bags are placed in an upright position on a warming plate at a temperature of 90–100° F. until the contents are thoroughly dry; usually 24 hours is sufficient. The leaflets from each bag are then ground to a fine powder in a mortar. One Gm. of the dry powder is carefully weighed out, placed in a small shell vial and covered with 5 cc. of acetone. If less than 1 Gm. of leaf powder is available, a correspondingly smaller amount of acetone is added. The vial is tightly stoppered, the contents are shaken, and extraction is allowed to continue overnight at room temperature. The next morning the vials are shaken again, the contents are allowed to settle and the desired amounts of extracts are pipetted off from above the leaf particles and added directly to the bottles containing fish. It has been found convenient to take samples from no more than 24 to 30 plants per day.

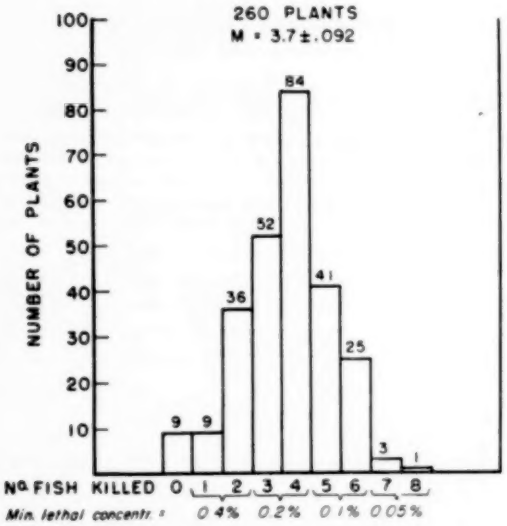

Fig. 3.—Distribution of marihuana potencies among 260 hemp plants.

Plants with a low active principle content kill a small number of fish (high minimum lethal concentration); those with a high active principle content kill a large number of fish (low minimum lethal concentration).

The use of cotton plugs and adapter tubes to remove the small particles of leaves, as suggested by Robinson, was found to be unnecessary if the vials were allowed to settle and if a pipette of small bore was used. The use of decolorizing charcoal was also discontinued when it was found that the potency of extracts apparently was reduced by such treatment.

RESULTS

Resin Gradient.—By using the method outlined above, in which as little as 0.75 cc. of extract is required for a test, it becomes feasible to assay single leaves. This made possible a study of the distribution of the marihuana resins in the different leaves of a single mature plant. Figure 1 shows the results of such an analysis. It will be noted that the first five leaves failed to kill fish, even in the strongest concentration (0.4%). There apparently is little or no activity in the lower leaves. From the sixth leaf up there is an irregular but definite increase in toxicity, until those near the top of the plant kill all fish, even in the 0.05% concentration.

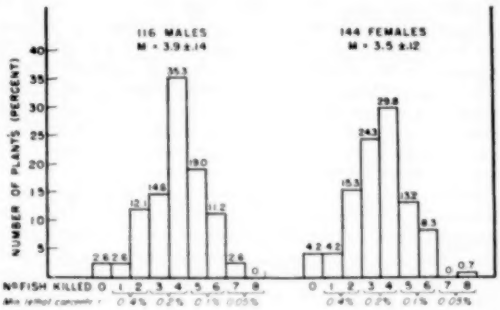

Fig. 4.—A comparison of activity of male and female hemp plants.

The mean is 3.9 for 116 males and 3.5 for 144 females, indicating little if any sex difference.

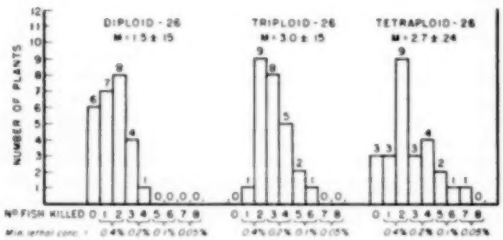

Fig. 5.—The relation of polyploidy to active resin content in hemp.

Twenty-six plants each from diploid, triploid and tetraploid pedigrees were tested. The triploids and tetraploids show a significantly higher toxicity than the diploids in these tests.

This would indicate at least an eightfold difference in activity between the lower and upper leaves of the same plant, the upper leaves being the more potent. Similar tests on other hemp plants indicate this to be a general rule.

Retests.—A valid method of assay must provide opportunity for retesting and must give consistant results when retests are made. Using the method described above, as many as five tests have been run on a single plant. The center leaflet is removed from each leaf in the initial test, and the next leaflet to right or left is removed in

successive tests. Each sample is ground, weighed and extracted separately.

Figure 2 shows graphically the results of retests on 8 different plants. In all cases, samples from a single plant were taken on the same day. The first of these plants was tested 5 times; two of the tests killed 2 fish, and the three others killed 3 fish each. The second plant was also tested 5 times, and all 5 of these tests killed 2 fish. The other plants were each tested twice, and in no case did the original test and the retest differ by more than 1 fish. This is an exceptionally small error, considering the nature of the assay.

Range in toxicity.—The results of the analysis of 260 mature plants are given in Fig. 3. It is of interest that these plants, although of the same variety and grown in the field under as nearly as possible identical conditions, vary widely in potency. The majority, of course, are intermediate in strength and kill from 2 to 6 fish (minimum lethal concentrations of 0.2% and 0.1%); but some plants were found with extremely low or extremely high activity. The 9 plants on the left of Fig. 3 failed to kill a single fish, even in the 0.4% concentration. At the other extreme, 1 plant was found whose extract killed all 8 fish; that is, through the 0.05% concentration.

These extremes represent a range in marihuana activity of over eightfold and are very encouraging from the breeding standpoint. The 9 plants with lowest activity include 3 males and 6 females. These have been intercrossed and should produce offspring with reduced average activity if this character is under genetic control.

Sex and toxicity.—The data in Fig. 3 were regrouped so as to give a comparison of active resins in male and female plants. The results are given in Fig. 4. It is to be noted that the range and distribution are very similar for males and females. The mean for the 116 males is 3.9, and the mean for the 144 females is 3.5. In all likelihood this difference is not significant, and tends to confirm the conclusions from previous limited chemical tests (1, 2, 3) that no important sex difference in activity exists. It is worthy of note, however, that these small differences in activity are in the same direction as those noted by Robinson and Matchett, the males being slightly higher in each case.

Polyploidy and Toxicity.—Determinations were also run on autopolyploid races of hemp, which had been developed at this laboratory with colchicine. The purpose of such tests was to determine whether marihuana activity is affected by increase in chromosome number. The polyploid races were derived from a single diploid variety; they have, therefore, a comparable but not necessarily an identical genetic background, since the original stock was not inbred. These plants were of a different variety than those reported on above and were grown under somewhat different conditions.

The results of assays on 26 diploids, 26 triploids and 26 tetraploids are given graphically in Fig. 5. The modal class is the same in each instance, but the ranges and means differ considerably. The mean of the diploids is 1.5, of the triploids 3.0 and of the tetraploids 2.7. Although made on a small number of plants, these tests seem to indicate a higher average activity in the triploid and tetraploid plants. The differences between the diploids and triploids and between the diploids and tetraploids are probably statistically significant (Diff./S. E. = 7.3 and 4.1, respectively); the difference between the triploid and tetraploid is not significant (Diff./S. E. = 1.4). The fact that the drug content is slightly greater in the triploid than in the tetraploid is out of line with the polyploid series; whether this difference will be borne out in more extensive testing is not known.

CONCLUSIONS

1. A modification of the Robinson bioassay is described. Relative activity is measured by a comparison of minimum lethal concentrations, and sampling is done by removing the center or adjacent leaflets from each leaf of a plant.

2. Retests of successive samples from the same plant show a high degree of reliability.

3. A gradient in activity of the different leaves of the same plant has been demonstrated. The bottom leaves have a low and the top leaves a high resin content.

4. At least an eightfold range in activity was found among plants of the same variety grown under similar cultural conditions.

5. Male and female plants appear to differ only slightly, if at all, in marihuana potency.

6. Assays on a limited number of plants indicate that experimental triploids and tetraploids have a significantly higher active principle than the diploids from which they were derived.

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Determination of Camphor and Alcohol in Spirit of Camphor by Means of Physical Constants, Optical Rotation and Specific Gravity*

By Elmer M. Plein† and Charles F. Poe

For several years a critical study has been made in this laboratory of the optical activity of camphor in alcoholic solutions (1) and of the various methods which might be used to determine the camphor and alcohol in spirit of camphor (2, 3). The method presented here makes use of two physical constants, optical rotation and specific gravity. Weber (4) and Schoorl (5) used physical constants, refractive index and specific gravity, for the analysis of samples of spirit of camphor which are official in the German and Dutch Pharmacopœias, respectively. Schoorl used also other pairs of physical constants. However, the works of Weber and Schoorl are not applicable to the analysis of the U. S. P. spirit because the preparations in the German and Dutch Pharmacopœias contain 10% (*w/w*) of camphor and about 60% (*w/w*) of alcohol. The composition of those spirits therefore varies considerably from that of the U. S. P. product.

TABLE 1.—THE ANALYSIS OF SPIRIT OF CAMPHOR BY MEANS OF SACCHARIMETER READING AND SPECIFIC GRAVITY

Camphor, %	Alcohol, %	Saccharimeter Reading	Specific Gravity	Camphor, % Determined	Variation, % Camphor	Alcohol, % Determined	Variation, % Alcohol
7.50	70.00	16.56	0.8780	7.55	+0.05	70.00	0.00
7.50	87.93	18.00	.8237	7.52	+0.02	87.85	−0.08
7.50	90.00	18.30	.8153	7.48	−0.02	90.00	0.00
7.85	77.00	17.70	.8580	7.85	0.00	77.05	+0.05
7.92	73.00	17.60	.8695	7.91	−0.01	73.00	0.00
8.00	86.00	19.01	.8297	8.01	+0.01	85.94	−0.06
8.75	78.00	19.80	.8538	8.68	−0.07	78.08	+0.08
8.80	74.00	19.68	.8658	8.77	−0.03	73.95	−0.05
8.90	80.00	20.47	.8479	8.88	−0.02	80.00	0.00
9.00	88.00	22.02	.8194	8.98	−0.02	88.02	+0.02
9.35	82.50	22.25	.8388	9.45	+0.10	82.50	0.00
9.40	83.00	22.20	.8365	9.38	−0.02	83.20	+0.20
9.75	87.50	23.88	.8199	9.70	−0.05	87.50	0.00
9.80	85.00	23.65	.8300	9.84	+0.04	84.96	−0.04
10.00	85.31	24.21	.8286	10.04	+0.04	85.24	−0.07
10.00	70.00	22.25	.8751	10.00	0.00	70.00	0.00
10.00	74.00	22.60	.8640	9.97	−0.03	74.00	0.00
10.00	80.00	23.25	.8467	10.00	0.00	79.86	−0.14
10.00	80.00	23.00	.8465	9.89	−0.11	80.00	0.00
10.00	80.50	23.39	.8440	10.02	+0.02	80.60	+0.10
10.00	86.00	24.09	.8250	9.89	−0.11	86.10	+0.10
10.00	85.31	24.16	.8285	10.01	+0.01	85.25	−0.06
10.00	85.31	24.11	.8280	9.97	−0.03	85.37	+0.06
10.00	85.31	24.08	.8287	9.96	−0.04	85.26	−0.05
10.20	83.00	24.36	.8364	10.24	+0.04	82.87	−0.13
10.60	76.00	24.25	.8566	10.59	−0.01	76.25	+0.25
10.80	83.00	25.69	.8351	10.72	−0.08	82.93	−0.07
10.90	82.00	26.04	.8375	10.91	+0.01	82.05	+0.05
11.00	75.00	24.99	.8601	10.92	−0.08	74.96	−0.04
11.00	84.00	27.00	.8300	11.12	+0.12	84.10	+0.10
11.10	87.00	27.70	.8188	11.10	0.00	86.95	−0.05
11.50	70.00	25.89	.8730	11.48	−0.02	70.04	+0.04
11.50	85.00	28.35	.8257	11.53	+0.03	85.10	+0.10

EXPERIMENTAL

The solutions studied were made to contain varying percentages of camphor, from 7.5% to 11.5% (*w/v*), and varying percentages of alcohol, from 70% to 90% (*v/v*), by the method described in a previous communication (3). High grade camphor which had been purified by sublimation was used. The first 10% and the last 5% of the sublimate were rejected.

The optical rotation of each solution was determined by means of a saccharimeter equipped with a Ventzke scale. All readings were taken in a 200-mm. tube at a temperature of 20° C. The specific gravities were determined with a 25-cc. pycnometer at 20/20° C.

From the optical rotations and the specific gravi-

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ties of about one hundred solutions a chart was constructed in which the ordinates represent the saccharimeter readings and the abscissas represent the specific gravities (Fig. 1). By connecting the points as plotted, several four-sided figures were formed and divided into smaller units to facilitate the use of the chart. Greater accuracy can be obtained by using an enlarged chart with additional subdivisions. The nearly horizontal lines represent the various camphor percentages from 7.5 to 11.5, and the nearly vertical lines represent the alcohol percentages from 70 to 90.

After the chart was constructed, it was used for the analysis of samples of spirit of camphor of known composition. Both the saccharimeter readings and the specific gravities were determined at 20° C. known composition were analyzed by use of a chart similar to the one given in Fig. 1. The results of these analyses, the determined values of which are in accord with the theoretical values, are given in Table I. The camphor content shows variations from −0.11 to +0.12% (average deviation, −0.01%) and the alcohol from −0.14 to +0.25% (average deviation, +0.01%).

CONCLUSIONS

1. A chart has been constructed from which the percentages of alcohol and camphor in spirit of camphor may be determined when the optical activity and specific gravity are known.

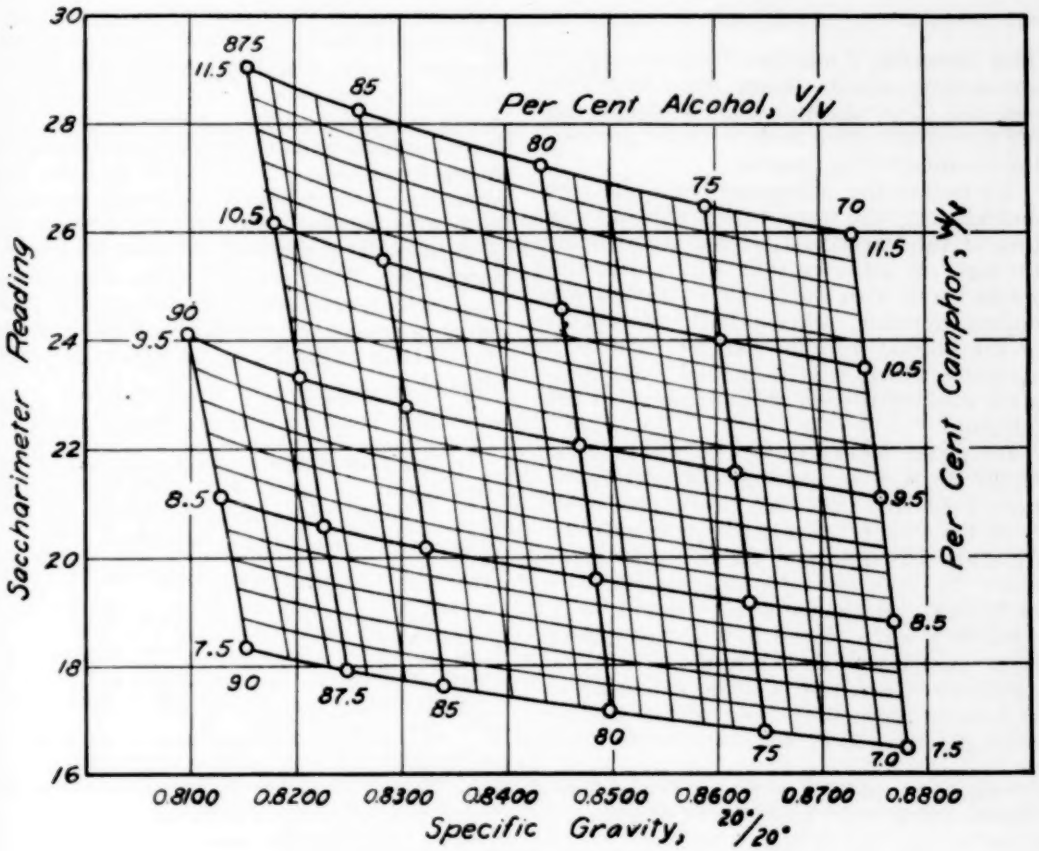

Fig. 1.

The point of intersection determined from these two constants is located by reference to the respective ordinate and abscissa. From this point of intersection, the percentage of alcohol is determined by following along the nearly vertical lines from the point of intersection to the outside of the four-sided figure, either to the top or bottom. The percentage of camphor likewise is determined by following the nearly horizontal lines to the outside of the figure, either to the right or left. For example, the saccharimeter reading of 24.0 and the specific gravity of 0.8400 may be taken. After the point of intersection has been found, the alcohol content can be read as 81.7% and the camphor content as 10.17%.

Thirty-three samples of spirit of camphor of

2. Analyses of different samples of spirit of camphor by use of the chart show close agreement with the theoretical values.

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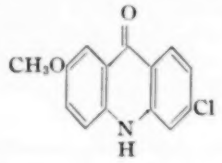
The Microscopic Identification of 2-Methoxy-6-Chloroacridone*

By George L. Keenan and Llewellyn H. Welsh

There frequently appear in commercial samples of quinacrine dihydrochloride small amounts of a water-insoluble impurity.



This impurity, 2-methoxy-6-chloroacridone, apparently results from the hydrolytic cleavage (1) of the quinacrine molecule during recrystallization from aqueous media in the manufacturing process.

To isolate the substance from the commercial drug, the warm turbid aqueous solution of the latter was filtered with suction through an asbestos mat which was then washed well with water and extracted with boiling pyridine. Steam bath evaporation of the filtered pyridine extract yielded the acridone which was examined directly or after washing it with small amounts of cold ethanol.

Literature reports (2, 3, 4) of the behavior of the acridone in melting point determinations suggested that such procedures would be of little value for identification purposes. Crystals of 2-methoxy-6-chloroacridone, prepared by hydrolyzing quinacrine dihydro- underwent no change other than a slight darkening when placed in a Fisher melting point apparatus and heated to 300° C. Although it was not possible to obtain information through the use of the melting point determination, microscopic identification of the substance could be made by a determination of its optical crystallographic properties. In ordinary light, the crystals consist of yellow rods and needles, some of which are curved. In parallel polarized light (crossed nicols), the extinction is parallel and the sign of elongation is negative. No interference figures were shown in convergent polarized light (crossed nicols). *Refractive indices:* $n_\alpha = 1.550$ (± 0.002), commonly shown when the rods and needles are oriented parallel to the vibration plane of the lower nicol: n_β and n_γ are both shown in the position at right angles (crosswise) to n_α and both are higher than the refractive index for methylene iodide (1.734). The frequent occurrence of the n_α value, however, together with the parallel extinction and negative elongation characterize the substance microscopically. The acridone was most conveniently recrystallized from pyridine, of which a somewhat smaller volume was required than of glacial acetic acid or isoamyl alcohol (2).

* Received from the U. S. Food & Drug Administration, Federal Security Agency, Washington, D. C.

SUMMARY

In the absence of suitable melting point characteristics, 2-methoxy-6-chloroacridone may be identified microscopically by its optical crystallographic properties.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Benzyloxy Carbonyl Chloride
t-Butyl lactate
Comenic acid or any ester thereof
Chelidonic acid or any ester thereof
Coproporphyrin
Dihydroxy maleic acid
7-Dehydrocholesterol (1 to 10 Kg.)
Diphosphor-glyceraldehyde
Epicatechin (3,5,7,3′,4′-flavanpentol)
Hydrocoerulignone
Inosine
Alpha-ketoglutaric acid
Phenyl acetone
1-(4-Amino-2-methyl)-5-pyrimidyl-2-methyl-3-(β-hydroxyethyl) pyridinium bromide hydrobromide (pyrithiamin)
Sodium amylopectin glycollate
Vanadium oxytrichloride